Genetic mechanisms of inherited bleeding disorders as a basis for personalised medicine approaches

GENETIC MECHANISMS OF INHERITED BLEEDING DISORDERS AS A BASIS FOR PERSONALISED MEDICINE APPROACHES

Alice Todaro

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CHAPTER 1

General introduction

HAEMOSTASIS

The word haemostasis is derived from ancient Greek and is composed of *haemo-*, meaning blood and *-stasis*, meaning to stop, to block, revealing the purpose of stopping the bleeding when a vascular injury occurs. Haemostasis is a finely regulated process comprising primary and secondary haemostasis (Figure 1). Primary haemostasis consists of the formation of the platelet plug in the damaged vessel to block blood loss. In secondary haemostasis, the coagulation cascade generates fibrin to stabilise the platelets plug. The two processes overlap in time and space and work together to seal the wound.

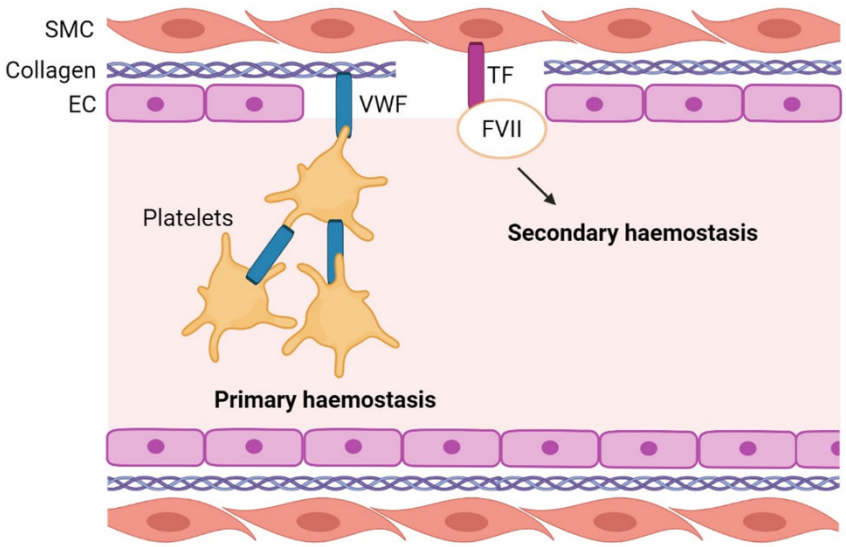

Figure 1. Simplified graphic representation of primary and secondary haemostasis. Damage of endothelial cells (EC) exposes collagen and smooth muscle cells (SMC) to the blood flow. The exposure of collagen promotes platelet activation and aggregation via von Willebrand factor (VWF) (primary haemostasis). The SMC display tissue factor (TF), a transmembrane protein that promotes the activation of coagulation factor VII (FVII) to FVIIa, initiating secondary haemostasis.

Primary haemostasis

Upon vascular injury, the sub-endothelium containing collagen and tissue factor-bearing cells is exposed to the blood flow. Platelet adhesion on the damaged surface is mediated by von Willebrand Factor (VWF) which creates a bridge between collagen in the sub-endothelium and GPIb and αIIbβ3 receptors on the platelet surface[1]. Platelets then undergo a shape change and start to aggregate and become activated, releasing a variety of coagulation factors and platelet agonists.

Secondary haemostasis

Secondary haemostasis consists of the coagulation cascade, a process in which coagulation factors are sequentially activated, eventually converting fibrinogen into fibrin[2]. Classically, the coagulation cascade is divided into intrinsic (or contact activation pathway) and extrinsic pathway. They merge into the common pathway at the level of activated factor X (FXa) (Figure 2).

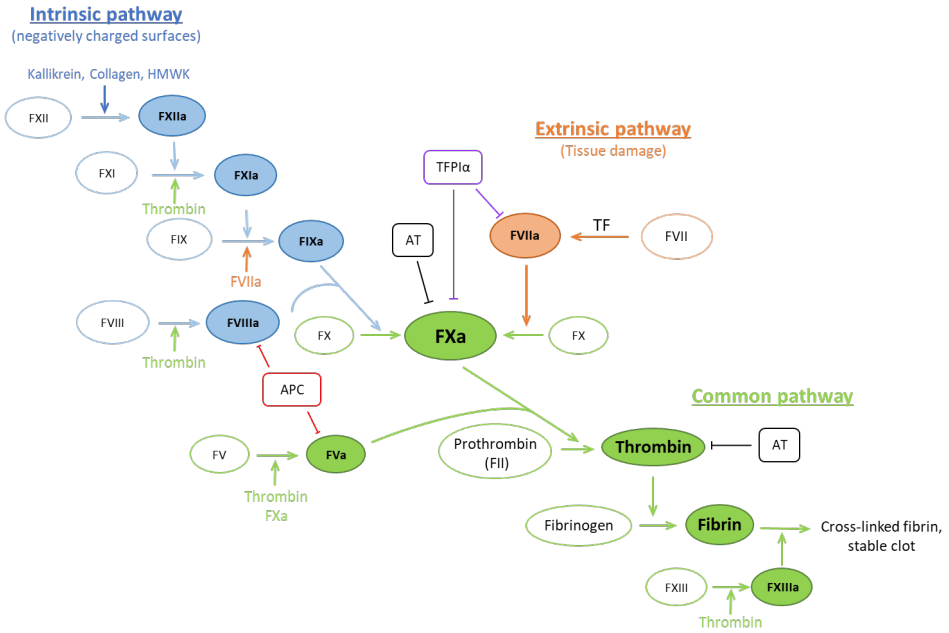

Figure 2. Coagulation cascade. Intrinsic (blue) and extrinsic (orange) pathways of coagulation cascade merge into the common pathway (green) to eventually form fibrin to stop blood loss after vascular damage. Tissue factor pathway inhibitor (TFPIα), Antithrombin (AT) and activated protein C (APC) are inhibitors of the coagulation cascade. HMWK = high molecular weight kininogen.

According to the prevalent cell-based model developed by Hoffman[3] the coagulation process occurs in three stages: initiation, amplification and propagation.

Initiation

At a site of vascular injury, the exposed tissue factor (TF) binds to factor VII (FVII) and promotes its proteolytic activation to FVIIa. The complex TF/FVIIa activates FX to activated FX (FXa). The latter activates coagulation factor V (FV) to FVa and combines with it to form the prothrombinase complex that converts prothrombin to thrombin, forming the initial traces of thrombin.

Amplification
The freshly generated thrombin activates factor XI (FXI -> FXIa), factor VIII (FVIII -> FVIIIa) and more FV -> FVa. FXIa is responsible for the activation of factor IX (FIX -> FIXa).

Propagation
During propagation, FIXa combines with its cofactor (FVIIIa) and its substrate (FX) in the tenase complex, on the surface of activated platelets. This complex activates FX into FXa. FXa then joins FVa and prothrombin on the platelet surface, thus forming the prothrombinase complex, which efficiently generates a large amount of thrombin. Finally, thrombin cleaves fibrinogen into fibrin which forms, together with platelets, a haemostatic plug at the wound site.

REGULATION OF COAGULATION

To prevent excessive clot formation, several inhibitors are in place to regulate and localise coagulation.

Activated protein C (APC)
Thrombin not only promotes the positive feedback for its own generation, but also activates the protein C anticoagulant pathway. In fact, thrombin binds thrombomodulin (TM) present on the surface of endothelial cells. The thrombin-TM complex activates protein C into activated protein C (APC) which, together with its cofactor protein S (PS), mediates the inactivation of FVa and FVIIIa[4].

Antithrombin (AT)
Antithrombin, a member of the serpin family, inhibits thrombin and FXa. The activity of AT is stimulated by heparin[5].

Tissue factor pathway inhibitor (TFPI)
TFPI is an anticoagulant protein that inhibits the early stages of coagulation. In humans, two alternatively spliced isoforms are present: TFPIα and TFPIβ[6]. TFPIα is present in endothelial cells, plasma and platelets and it is composed by a N-terminal region, three Kunitz domains and a C-terminal (C-term) region[7]. The first and second Kunitz domains inhibit the TF/FVIIa complex and FXa, respectively. The third Kunitz domain binds protein S (PS), which serves as cofactor of TFPIα to enhance the inhibition of FXa[8,9]. The C-term of TFPIα, binds the acidic region in the B-domain of factor V (FV)/ factor V short (FV-short)[10]. TFPIβ is expressed on endothelial cells and has the same Kunitz domains 1 and 2 as TFPIα, and a C-term encoding a glycosylphosphatidylinositol (GPI) anchor binding the endothelial cell surface[11].

BLEEDING

Bleeding disorders can be caused by acquired or inherited defects in primary or secondary haemostasis, such as platelet function defects or deficiencies of coagulation factors. Acquired bleeding disorders can be caused by the development of auto-antibodies, liver diseases, renal diseases, or anticoagulant therapy[12]. Inherited bleeding disorders are caused by genetic mutations affecting coagulation factors or proteins involved in platelet formation and/or function. The most common inherited bleeding disorders are von Willebrand disease (VWD) and haemophilia A (FVIII deficiency) and B (FIX deficiency)[13]. Rare bleeding disorders include deficiencies of fibrinogen, prothrombin, FV, FVII, combined FV and FVIII, FX, FXI and FXIII[14]. The severity of defect is usually classified based on the residual activity of the protein involved and it is divided in severe, moderate and mild. The same classification is applied for the bleeding symptoms. Clinical manifestations of bleeding disorders include epistaxis, easy bruising, prolonged bleeding after surgery, delivery or minor injuries, menorrhagia in women, and rare life threatening symptoms as gastrointestinal and intracranial bleeding[15]. This thesis focusses on bleeding disorders caused by genetic defects of VWF and FV.

Von Willebrand factor (VWF)

Von Willebrand factor plays a role in both primary and secondary haemostasis by supporting platelet adhesion and aggregation at sites of vascular injury and by acting as plasma carrier for FVIII[16]. VWF is a large multimeric protein encoded by the *VWF* gene located on chromosome 12. The *VWF* gene extends for ~178 kb and contains 52 exons. The mature VWF mRNA is 8.8-kb long[17]. VWF is synthesised by endothelial cells and megakaryocytes as a 2813-amino-acid (aa) monomeric pre-pro-VWF consisting of a signal peptide (22 aa), a propeptide (741 aa) and mature VWF (2050 aa) corresponding to 270 kDa, illustrated in Figure 3[18].

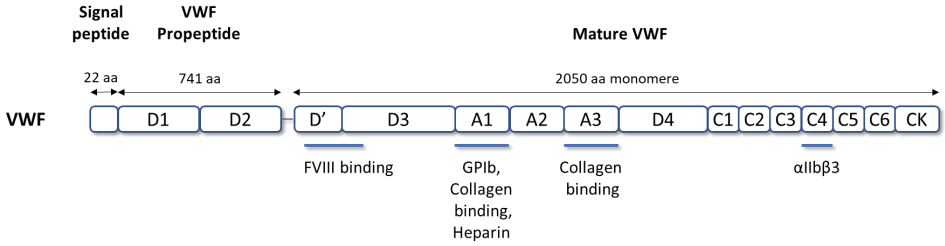

Figure 3. Schematic representation of VWF domains and binding sites.

VWF has a complex biosynthesis involving several post-translational modifications in the endoplasmic reticulum and Golgi apparatus, most prominently multimerization. In endothelial cells VWF is stored in the Weibel-Palade bodies (WPB) or released in the circulation. In plasma, the ultra-large multimers of VWF are cleaved into smaller multimers by ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13), which reduces the thrombotic risk[19]. The VWF/FVIII complex in plasma is crucial for FVIII stabilisation, protecting it from premature proteolysis and clearance[20].

In megakaryocytes, VWF is transported into platelet α-granules and released upon platelet activation[19]. Platelet VWF is rich in high molecular weight multimers[21], that are more efficient in platelet aggregation[22].

Von Willebrand disease (VWD)

Genetic variants in the *VWF* gene can lead to VWD, the most common inherited bleeding disorder with an estimated prevalence of 0.6-1.3% in the general population (https://www.orpha.net, October 2023). VWF defects can be quantitative (type 1 and 3 VWD) or qualitative (type 2 VWD)[23]. Type 1 VWD accounts for ~85% of cases and it is characterised by a partial deficiency of VWF. VWD type 3 is the least common (about 3%) and it is the most severe form of VWD, characterised by undetectable VWF. VWD type 2 is subclassified into four subtypes characterised by defects in multimerization (2A), increased platelet binding (2B), decreased platelet binding or collagen binding (2M) and decreased FVIII binding (2N), respectively[24]. The bleeding symptoms range from easy bruising, nose bleeding and heavy menstrual bleeding in women, to prolonged bleeding after injury, childbirth or surgery. Treatment options to prevent bleeding in VWD patients are: a) desmopressin administration, which stimulates the release of endogenous VWF from endothelial cells; b) infusion of exogenous VWF concentrate or combined FVIII-VWF concentrate; and c) antifibrinolytic drugs to prevent clot lysis [23,25]. Additionally, in women suffering from VWD, estrogen-containing oral contraceptives have shown efficacy in reducing menstrual blood loss[26].

Factor V

Coagulation factor V (FV) is a multidomain (A1-A2-B-A3-C1-C2) protein encoded by the *F5* gene on chromosome 1. The *F5* gene spans ~80 kb[27] and the 6.8-kb-long mature mRNA contains 25 exons[28]. FV is physiologically produced and secreted by hepatocytes and circulates as a single-chain inactive procofactor of 330 kDa[29]. FV in blood is distributed between two pools: approximately 80% is found in plasma, whereas the remaining 20% is found within the α-granules of platelets. The inactive state of FV is maintained by a high-affinity interaction between two evolutionary conserved sequences called basic region (BR) (residues 963-1008) and acidic region

(AR) (residues 1493-1537), both within the B domain. Proteolytic removal of the B domain by FXa and/or thrombin produces activated FV (FVa), which consists of a heavy chain (A1-A2) bound to a light chain (A3-C1-C2) through a calcium ion. Once activated, FVa functions as cofactor of FXa in the prothrombinase complex to convert prothrombin to thrombin[30]. FVa is a potent cofactor of FXa, and the presence of FVa in the prothrombinase complex accelerates the conversion of prothrombin to thrombin more than 1000-fold[31]. FVa is inactivated by APC by proteolytic cleavage of the heavy chain[32]. FV also acts as cofactor of APC for the inhibition of FVIIIa[30].

Alternative splicing of the *F5* gene: FV-short

The *F5* gene undergoes alternative splicing, a process that can select different exons within the same pre-mRNA resulting in different protein isoforms. In the case of *F5* the main splicing product (>95%) is FV, also called full-length FV in this thesis, while the minor product is known as FV-short[33] (Figure 4).

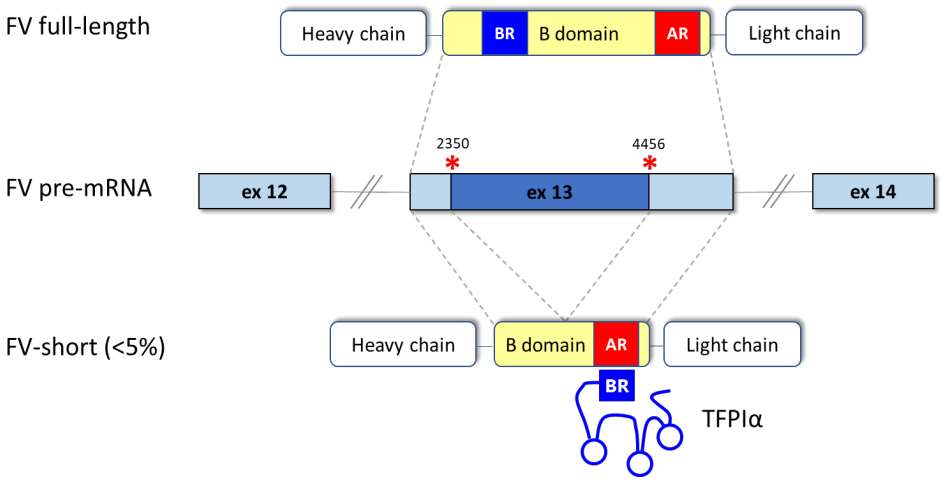

Figure 4. Schematic representation of FV and FV-short. The large *F5* exon 13 is normally retained in the mature mRNA, generating full-length FV. In less than 5% of transcripts, alternative splicing sites within exon 13 (marked with red asterisks) are used and the central part of exon 13 is spliced out. At the protein level, this results in the in-frame deletion of 702 aa including the basic region (BR), leading to the formation of FV-short. The C-terminal end of TFPIα contains a BR that binds the unmatched AR of FV-short with high affinity conferring anticoagulant properties to FV-short. BR: basic region; AR: acidic region; ex: exon. The mRNA positions and cleavage sites are expressed according to the HGVS nomenclature.

FV-short splicing uses an alternative donor (at nucleotide position 2350) and acceptor (at nucleotide position 4456) splice sites located within exon 13, removing 2106 nucleotides and resulting in the loss of 702 amino acids (residues 784-1486; legacy

nomenclature 756-1458) from the B domain. The alternative splicing event removes the BR, whereas the AR is retained. FV-short is considered the main carrier of plasma TFPIα, stabilising it in circulation and protecting it from truncation and clearance[33,34]. This tight interaction is mediated by the unmatched AR of FV-short and a basic region in the C-term of TFPIα (homologous to the BR of FV)[33]. The FV-short/TFPIα interaction, together with PS, also results in the inhibition of the constitutive prothrombinase activity of FV-short and in the inhibition of FXa[35,36]. FV-short was originally discovered in 2013 in an American family with an unexplained bleeding disorder[33], due to a mutation enhancing FV-short expression (see below).

BLEEDING DUE TO *F5* GENE MUTATIONS

Alterations in the *F5* gene can increase the risk of bleeding by causing FV deficiency or by up-regulating the expression of FV-short.

FV deficiency

Congenital FV deficiency is an autosomal recessive rare bleeding disorder with an estimated incidence of 1-9 per million (https://www.orpha.net, October 2023).

Efthymiou et al[37] recently reviewed the literature on *F5* genetic variation. They identified 199 unique variants associated with FV deficiency, classified as in Figure 5.

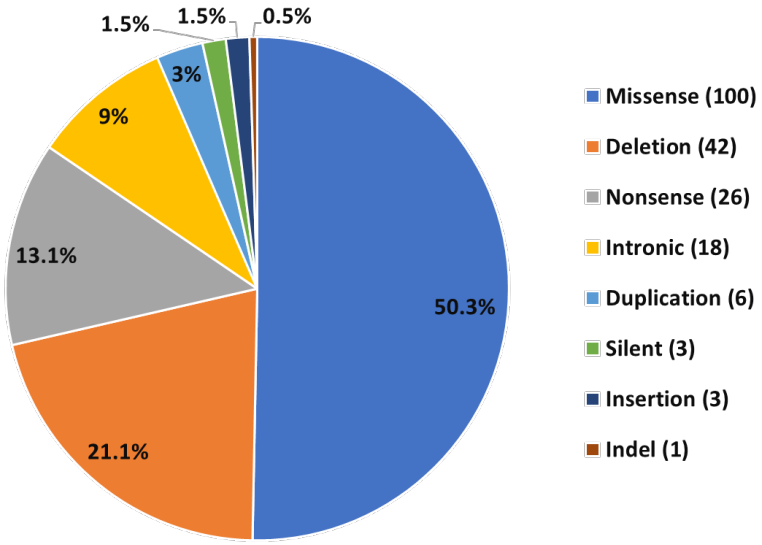

Figure 5. Distribution of *F5* mutations causing FV deficiency.

Based on plasma FV activity, FV deficiency is classified as severe (<1% of FV activity), moderate (1-5%) or mild (6-10%). Levels of FV below 1% may be associated with severe bleeding symptoms. However, many patients with very low or undetectable FV levels experience only moderate bleeding symptoms. This is the result of two modulators of bleeding tendency in FV deficiency: platelet FV and TFPIα. In particular, traces of residual FV in platelets[38] and constitutively low levels of TFPIα[39] often make it possible for FV-deficient patients to achieve minimal haemostasis, protecting them from life-threatening bleeding.

Heterozygous carriers of mutations causing FV deficiency are generally asymptomatic, whereas homozygotes and compound heterozygotes present a broad variety of symptoms, ranging from easy bruising, gum bleeding, epistaxis, haemarthrosis, haematomas, prolonged or excessive bleeding after surgery or trauma to life-threatening symptoms such as gastrointestinal and intra-cranial bleeding[40]. Women suffering of FV deficiency often experience menorrhagia and are exposed to a higher risk of miscarriage and post-partum haemorrhage[41]. Since a FV concentrate is not yet available for clinical use, FV-deficient patients are usually managed with fresh frozen plasma, antifibrinolytics or platelet concentrates[40]. Although effective, therapies based on plasma and platelet concentrates might expose patients to risks such as circulatory overload, infections, allergic reactions and TRALI (transfusion-related acute lung injury)[42]. The lack of a targeted therapy has stimulated the search for new and personalised therapeutic approaches with the goal to ameliorate the condition and the quality of life of FV-deficient patients.

Bleeding disorders related to FV-short

Currently, three genetic variants associated with pathological up-regulation of FV-short are known (FV-East Texas, FV-Amsterdam and FV-Atlanta).

FV-short was discovered nearly 10 years ago in an American family from East Texas with an unexplained bleeding disorder[33,43] and high plasma TFPIα levels (10-fold increase). This family segregates an autosomal dominant mutation (c.2350A>G) in exon 13 of the *F5* gene strengthening the donor splice site of the FV-short-specific intron and resulting in overexpression of FV-short (FV-East Texas). Recently, an apparently unrelated family from Indiana was found to segregate exactly the same mutation[44].

FV-Amsterdam, found in heterozygosity in a Dutch family, is the product of a nucleotide change (c.2588C>G) in exon 13 of the *F5* gene, resulting in the creation of a new donor splicing site for FV-short with an in-frame deletion of 623 amino acids within the B domain. FV-Amsterdam expression is also associated with increased TFPIα level (~12-fold increase)[45].

FV-Atlanta is a heterozygous deletion (*F5*: c.2413_3244del) of 832 bp in exon 13 of the *F5* gene, resulting in a frameshift with the introduction of a premature stop codon which abolishes the expression of full-length FV[46]. The deletion is spliced out in FV-short and therefore it does not compromise FV-short production. Interestingly, the deletion greatly enhances the expression of FV-short, suggesting the presence of an important regulatory sequence for FV-short splicing in the deleted region. The overexpression of FV-short results in a remarkably elevated level of TFPIα (22-fold), leading to a bleeding tendency.

MOLECULAR APPROACHES TO CORRECT MUTATIONS

In recent years, molecular approaches have been developed to target specific types of mutations responsible for genetic disorders.

Missense mutations introduce an amino acid change with a potentially detrimental effect on protein folding, secretion and/or function. Molecules, called chaperones, normally assist folding of nascent proteins. Small molecules that exhibit chaperone-like activity have shown the ability to rescue proteins hampered by missense mutations[47].
Nonsense mutations introduce a premature termination codon (PTC) in the mRNA sequence resulting in a truncated protein, which is often not functional or degraded intracellularly. A rare physiological process, called natural readthrough, may incorporate an amino acid at the PTC, thus leading to the formation of a full-length protein[48]. This process can be pharmacologically enhanced by small molecules known as readthrough agents, providing a strategy to rescue the synthesis of proteins impaired by nonsense mutations [49,50]. An example of this application is presented in this thesis.
Point mutations, insertions or deletions can cause mis-splicing by introducing or weakening a splice site, thus leading to the production of aberrant mRNAs which can be degraded or encode defective proteins[51]. In this thesis, two molecular correction approaches, illustrated in Figure 5, have been employed to change the ratio between alternatively spliced isoforms: morpholino antisense oligonucleotides (MAOs) and small interfering RNA (siRNAs).

Morpholino antisense oligonucleotides (MAO) are small, uncharged analogues of nucleic acids[52] that anneal to the target sequence by complementary base-pairing. MAOs can act on the pre-mRNA by binding a donor or an acceptor splice signal, masking the undesirable splice site and thus influencing the spliceosome decision[53].
Short interfering RNAs (siRNAs) are double-stranded siRNA composed by a guide (nonsense) and a passenger (sense) strand. Upon intracellular entrance, the siRNA is

incorporated in a protein complex called RISC (RNA-induced silencing complex) where it is activated (the passenger strand is removed, whereas the guide strand is retained). Once activated, the siRNA recognises the mRNA target sequence by complementary base pairing and induces its cleavage, thus preventing its translation[54].

Finally, gene editing technology has rapidly developed especially in recent years, providing many tools to modify the gene sequence and expression, such as CRISPR/Cas, prime editing and many others[55].

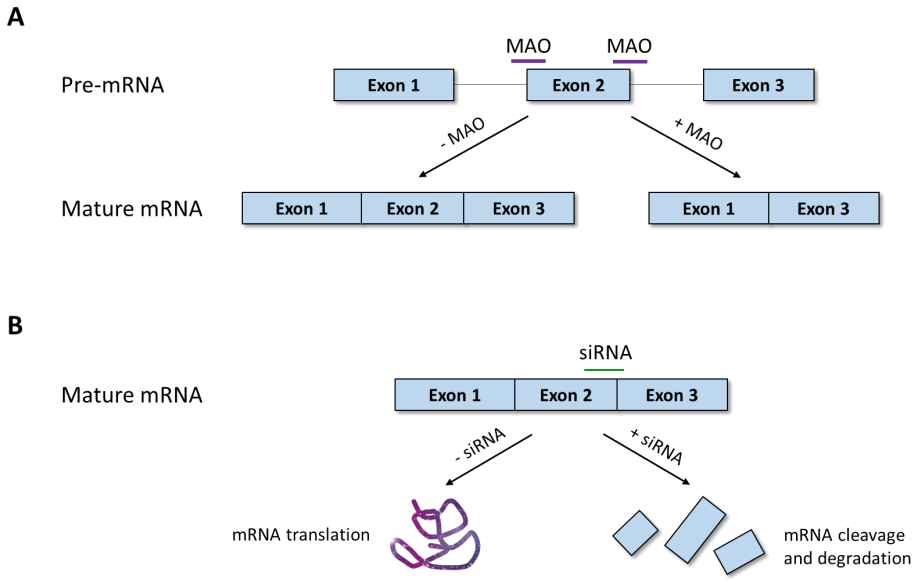

Figure 5. Schematic mechanism of action of MAO and siRNA. (A) MAOs bind to the target sequence on the pre-mRNA to block the splicing event on the selected donor/acceptor splice sites. This results in the skipping of the undesired sequence in the mature mRNA**. (B)** The mechanism of action of siRNAs is based on mature mRNA targeting. siRNA binds to the mRNA, hence promoting mRNA cleavage and degradation by intracellular RNAses.

OUTLINE OF THE THESIS

This thesis concerns inherited bleeding disorders caused by mutations in *VWF* (VWD) and *F5* (FV deficiency and FV-short-associated disorders) and presents possible molecular correction approaches to restore impaired FV production or to modulate FV-short splicing *in vitro*.

Chapter 2 investigates a patient with congenital von Willebrand disease. A novel genetic variant in *VWF* was discovered resulting in the impairment of VWF propeptide cleavage and affecting VWF-FVIII binding. The new variant was expressed in a recombinant system and functionally characterised *in vitro*.

Chapter 3 is based on a family with FV deficiency due to a nonsense mutation in the *F5* gene. Several compounds enhancing the natural ribosome readthrough process were tested to evaluate their ability to rescue FV synthesis and activity in an *in vitro* model of the mutation. The same compounds were also tested in an *ex vivo* model based on the patient's *ex vivo* differentiated megakaryocytes.

Chapter 4 presents an approach to downregulate FV-short. We designed and tested Morpholino Antisense Oligonucleotides (MAO) specifically targeting FV-short splicing to down-regulate its expression in a liver cell line that naturally expresses FV and FV-short. Since an assay to measure FV-short is not yet available, this chapter also describes the development of a real-time qPCR-based method to quantify FV-short mRNA.

In **chapter 5** a FV-short down-regulation approach using siRNA is presented and tested in *in vitro* models of the *F5* mutations that up-regulate FV-short expression (FV-East Texas, FV-Amsterdam and FV-Atlanta).

Chapter 6 integrates all findings of this thesis and discusses them in a broader context.

REFERENCES

1. Bryckaert, M., et al., *Of von Willebrand factor and platelets.* Cell Mol Life Sci, 2015. **72**(2): p. 307-26.
2. Winter, W.E., S.D. Flax, and N.S. Harris, *Coagulation Testing in the Core Laboratory.* Lab Med, 2017. **48**(4): p. 295-313.
3. Hoffman, M., *A cell-based model of coagulation and the role of factor VIIa.* Blood Rev, 2003. **17 Suppl 1**: p. S1-5.
4. Dahlbäck, B. and B.O. Villoutreix, *The anticoagulant protein C pathway.* FEBS Lett, 2005. **579**(15): p. 3310-6.
5. Corral, J., M.E. de la Morena-Barrio, and V. Vicente, *The genetics of antithrombin.* Thromb Res, 2018. **169**: p. 23-29.
6. Mast, A.E., *Tissue Factor Pathway Inhibitor: Multiple Anticoagulant Activities for a Single Protein.* Arterioscler Thromb Vasc Biol, 2016. **36**(1): p. 9-14.
7. Wood, J.P., et al., *Biology of tissue factor pathway inhibitor.* Blood, 2014. **123**(19): p. 2934-43.
8. Hackeng, T.M., et al., *Protein S stimulates inhibition of the tissue factor pathway by tissue factor pathway inhibitor.* Proc Natl Acad Sci U S A, 2006. **103**(9): p. 3106-11.
9. Ndonwi, M. and G. Broze, Jr., *Protein S enhances the tissue factor pathway inhibitor inhibition of factor Xa but not its inhibition of factor VIIa-tissue factor.* J Thromb Haemost, 2008. **6**(6): p. 1044-6.
10. Dahlbäck, B., *Natural anticoagulant discovery, the gift that keeps on giving: finding FV-Short.* J Thromb Haemost, 2023. **21**(4): p. 716-727.
11. Girard, T.J., E. Tuley, and G.J. Broze, Jr., *TFPIβ is the GPI-anchored TFPI isoform on human endothelial cells and placental microsomes.* Blood, 2012. **119**(5): p. 1256-62.
12. Hurwitz, A., R. Massone, and B.L. Lopez, *Acquired Bleeding Disorders.* Hematol Oncol Clin North Am, 2017. **31**(6): p. 1123-1145.
13. Doherty, T.M. and A. Kelley, *Bleeding Disorders*, in *StatPearls*. 2023, StatPearls Publishing Copyright © 2023, StatPearls Publishing LLC.: Treasure Island (FL).
14. Dorgalaleh, A., et al., *Molecular basis of rare congenital bleeding disorders.* Blood Rev, 2023. **59**: p. 101029.
15. Brown, D.L., *Congenital bleeding disorders.* Curr Probl Pediatr Adolesc Health Care, 2005. **35**(2): p. 38-62.
16. Peyvandi, F., I. Garagiola, and L. Baronciani, *Role of von Willebrand factor in the haemostasis.* Blood Transfus, 2011. **9 Suppl 2**(Suppl 2): p. s3-8.
17. Mancuso, D.J., et al., *Structure of the gene for human von Willebrand factor.* J Biol Chem, 1989. **264**(33): p. 19514-27.
18. Verweij, C.L., et al., *Full-length von Willebrand factor (vWF) cDNA encodes a highly repetitive protein considerably larger than the mature vWF subunit.* Embo j, 1986. **5**(8): p. 1839-47.
19. Lenting, P.J., O.D. Christophe, and C.V. Denis, *von Willebrand factor biosynthesis, secretion, and clearance: connecting the far ends.* Blood, 2015. **125**(13): p. 2019-28.
20. Pipe, S.W., et al., *Life in the shadow of a dominant partner: the FVIII-VWF association and its clinical implications for hemophilia A.* Blood, 2016. **128**(16): p. 2007-2016.
21. McGrath, R.T., et al., *Platelet von Willebrand factor--structure, function and biological importance.* Br J Haematol, 2010. **148**(6): p. 834-43.
22. Stockschlaeder, M., R. Schneppenheim, and U. Budde, *Update on von Willebrand factor multimers: focus on high-molecular-weight multimers and their role in hemostasis.* Blood Coagul Fibrinolysis, 2014. **25**(3): p. 206-16.
23. Leebeek, F.W. and J.C. Eikenboom, *Von Willebrand's Disease.* N Engl J Med, 2016. **375**(21): p. 2067-2080.
24. Favaloro, E.J., *Classification of von Willebrand disease in the context of modern contemporary von*

Willebrand factor testing methodologies. Res Pract Thromb Haemost, 2020. **4**(6): p. 952-957.

25. Curnow, J., L. Pasalic, and E.J. Favaloro, *Treatment of von Willebrand Disease.* Semin Thromb Hemost, 2016. **42**(2): p. 133-46.

26. Weyand, A.C. and V.H. Flood, *Von Willebrand Disease: Current Status of Diagnosis and Management.* Hematol Oncol Clin North Am, 2021. **35**(6): p. 1085-1101.

27. Cripe, L.D., K.D. Moore, and W.H. Kane, *Structure of the gene for human coagulation factor V.* Biochemistry, 1992. **31**(15): p. 3777-85.

28. Jenny, R.J., et al., *Complete cDNA and derived amino acid sequence of human factor V.* Proc Natl Acad Sci U S A, 1987. **84**(14): p. 4846-50.

29. Camire, R.M. and M.H. Bos, *The molecular basis of factor V and VIII procofactor activation.* J Thromb Haemost, 2009. **7**(12): p. 1951-61.

30. Dahlbäck, B., *Pro- and anticoagulant properties of factor V in pathogenesis of thrombosis and bleeding disorders.* Int J Lab Hematol, 2016. **38 Suppl 1**: p. 4-11.

31. Rosing, J., et al., *The role of phospholipids and factor Va in the prothrombinase complex.* J Biol Chem, 1980. **255**(1): p. 274-83.

32. Dahlbäck, B., *Novel insights into the regulation of coagulation by factor V isoforms, tissue factor pathway inhibitorα, and protein S.* J Thromb Haemost, 2017. **15**(7): p. 1241-1250.

33. Vincent, L.M., et al., *Coagulation factor V(A2440G) causes east Texas bleeding disorder via TFPIα.* J Clin Invest, 2013. **123**(9): p. 3777-87.

34. Dahlbäck, B., et al., *Factor V-short and protein S as synergistic tissue factor pathway inhibitor (TFPIα) cofactors.* Res Pract Thromb Haemost, 2018. **2**(1): p. 114-124.

35. Petrillo, T., et al., *Regulation of factor V and factor V-short by TFPIα: Relationship between B-domain proteolysis and binding.* J Biol Chem, 2021. **296**: p. 100234.

36. Dahlbäck, B. and S. Tran, *The preAR2 region (1458-1492) in factor V-Short is crucial for the synergistic TFPIα-cofactor activity with protein S and the assembly of a trimolecular factor Xa-inhibitory complex comprising FV-Short, protein S, and TFPIα.* J Thromb Haemost, 2022. **20**(1): p. 58-68.

37. Efthymiou, C., et al., *Analysis of 363 Genetic Variants in F5 via an Interactive Web Database Reveals New Insights into FV Deficiency and FV Leiden.* TH Open, 2023. **7**(1): p. e30-e41.

38. Duckers, C., et al., *Residual platelet factor V ensures thrombin generation in patients with severe congenital factor V deficiency and mild bleeding symptoms.* Blood, 2010. **115**(4): p. 879-86.

39. Duckers, C., et al., *Low plasma levels of tissue factor pathway inhibitor in patients with congenital factor V deficiency.* Blood, 2008. **112**(9): p. 3615-23.

40. Asselta, R. and F. Peyvandi, *Factor V deficiency.* Semin Thromb Hemost, 2009. **35**(4): p. 382-9.

41. Naderi, M., et al., *Miscarriage and recurrent miscarriage in patients with congenital factor V deficiency: a report of six cases in Iran.* Int J Hematol, 2016. **103**(6): p. 673-5.

42. Pandey, S. and G.N. Vyas, *Adverse effects of plasma transfusion.* Transfusion, 2012. **52 Suppl 1**(Suppl 1): p. 65s-79s.

43. Kuang, S.Q., et al., *Characterization of a novel autosomal dominant bleeding disorder in a large kindred from east Texas.* Blood, 2001. **97**(6): p. 1549-54.

44. Peterson, J.A., et al., *Factor V east Texas variant causes bleeding in a three-generation family.* J Thromb Haemost, 2022. **20**(3): p. 565-573.

45. Cunha, M.L., et al., *A novel mutation in the F5 gene (factor V Amsterdam) associated with bleeding independent of factor V procoagulant function.* Blood, 2015. **125**(11): p. 1822-5.

46. Zimowski, K.L., et al., *F5-Atlanta: A novel mutation in F5 associated with enhanced East Texas splicing and FV-short production.* J Thromb Haemost, 2021. **19**(7): p. 1653-1665.

47. Yadav, K., et al., *Protein Misfolding Diseases and Therapeutic Approaches.* Curr Protein Pept Sci, 2019. **20**(12): p. 1226-1245.

48. Rospert, S., M. Rakwalska, and Y. Dubaquié, *Polypeptide chain termination and stop codon readthrough on eukaryotic ribosomes.* Rev Physiol Biochem Pharmacol, 2005. **155**: p. 1-30.

49. Keeling, K.M. and D.M. Bedwell, *Suppression of nonsense mutations as a therapeutic approach to treat genetic diseases.* Wiley Interdiscip Rev RNA, 2011. **2**(6): p. 837-52.

50. Lombardi, S., et al., *Molecular Insights into Determinants of Translational Readthrough and Implications for Nonsense Suppression Approaches.* Int J Mol Sci, 2020. **21**(24).

51. Abramowicz, A. and M. Gos, *Correction to: Splicing mutations in human genetic disorders: examples, detection, and confirmation.* J Appl Genet, 2019. **60**(2): p. 231.

52. Summerton, J. and D. Weller, *Morpholino antisense oligomers: design, preparation, and properties.* Antisense Nucleic Acid Drug Dev, 1997. **7**(3): p. 187-95.

53. Havens, M.A. and M.L. Hastings, *Splice-switching antisense oligonucleotides as therapeutic drugs.* Nucleic Acids Res, 2016. **44**(14): p. 6549-63.

54. Friedrich, M. and A. Aigner, *Therapeutic siRNA: State-of-the-Art and Future Perspectives.* BioDrugs, 2022. **36**(5): p. 549-571.

55. Huang, S., et al., *Research progress in gene editing technology.* Front Biosci (Landmark Ed), 2021. **26**(10): p. 916-927.

CHAPTER 2

Novel von Willebrand factor propeptide variant (R760S) in a patient with combined type 1/type 2N von Willebrand disease

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CHAPTER 3

In vitro and *ex vivo* rescue of a nonsense mutation responsible for severe coagulation factor V deficiency

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ABSTRACT

Background
Coagulation factor V (FV) deficiency is a rare bleeding disorder that is usually managed with fresh-frozen plasma. Patients with nonsense mutations may respond to treatment with readthrough agents.

Objective
To investigate whether the *F5* p.Arg1161Ter mutation, causing severe FV deficiency in several patients, would be amenable to readthrough therapy.

Methods
F5 mRNA and protein expression was evaluated in a *F5* p.Arg1161Ter-homozygous patient. Five readthrough agents with different mechanisms of action, i.e. G418, ELX-02, PTC-124, 2,6-diaminopurine (2,6-DAP) and Amlexanox (AMX), were tested in *in vitro* and *ex vivo* models of the mutation.

Results
The *F5* p.Arg1161Ter-homozygous patient showed residual *F5* mRNA and functional platelet FV, indicating detectable levels of natural readthrough. COS-1 cells transfected with the FV-Arg1161Ter cDNA expressed 0.7% FV activity compared to wild-type. Treatment with 0-500 μM G418, ELX-02 and 2,6-DAP dose-dependently increased FV activity up to 7.0-fold, 3.1-fold and 10.8-fold, respectively, whereas PTC-124 and AMX (alone or in combination) were ineffective. These findings were confirmed by thrombin generation assays in FV-depleted plasma reconstituted with conditioned media of treated cells. All compounds except ELX-02 showed some degree of cytotoxicity. *Ex-vivo* differentiated megakaryocytes of the *F5* p.Arg1161Ter-homozygous patient, which were negative at FV immunostaining, turned positive after treatment with all five readthrough agents. Notably, they were also able to internalise mutant FV rescued with G418 or 2,6-DAP, which would be required to maintain the crucial platelet FV pool *in vivo*.

Conclusions
These findings provide *in vitro* and *ex vivo* proof-of-principle for readthrough-mediated rescue of the *F5* p.Arg1161Ter mutation.

Keywords
Factor V deficiency, bleeding, nonsense mutation, nonsense-mediated decay, translational readthrough, megakaryocytes

INTRODUCTION

Coagulation factor V (FV)[1], encoded by the *F5* gene, is a liver-derived multi-domain glycoprotein (A1-A2-B-A3-C1-C2) present both in plasma (80%) and in platelet α-granules (20%). Its activated form (FVa) accelerates factor Xa (FXa)-catalysed conversion of prothrombin to thrombin >1000-fold[2], making FV indispensable to life[3]. Congenital FV deficiency is a rare autosomal recessive bleeding disorder of variable severity[4], ranging from mucosal and post-traumatic bleeding to life-threatening intracranial haemorrhages[5]. The main determinant of bleeding tendency is the level of residual platelet FV, traces of which are usually sufficient to guarantee minimal haemostasis[6]. In fact, platelet FV, which originates from endocytosis and intracellular processing of plasma FV by bone-marrow megakaryocytes[7], has enhanced procoagulant properties[8]. Moreover, circulating tissue factor pathway inhibitor (TFPIα), a direct inhibitor of FV activation[9] and early prothrombinase complexes[10], is constitutively low in FV deficiency[11], because FV and particularly its minor splicing isoform FV-short[12] are required to maintain TFPIα in the circulation[11]. Since no FV concentrate is clinically available[13], the treatment and prophylaxis of FV-deficient patients still relies on fresh-frozen plasma[14]. However, alternative therapeutic approaches are being explored[15,16], including molecular strategies targeting specific genetic defects[17-19]. Nonsense mutations, which introduce a premature termination codon (PTC), represent ~13% (26/199) of the FV deficiency mutational spectrum[20]. These mutations are usually considered null defects, because PTCs trigger mRNA degradation by the nonsense-mediated decay (NMD) pathway[21] and/or result in truncated non-functional proteins. However, a fraction of the PTC-containing mRNA actually escapes NMD as the PTC is mistakenly translated into an amino acid, allowing the synthesis of tiny amounts of full-length protein ("translational readthrough")[22,23]. The efficiency of this rescue mechanism depends on the position, identity and sequence context of the PTC, and can be pharmacologically enhanced by small molecules known as readthrough agents[23,24]. The latter include aminoglycoside antibiotics, like G418 (Geneticin)[25] and its synthetic analogue ELX-02[26,27], which alter the ability of the eukaryotic ribosome to decode stop codons; PTC-124 (Ataluren), which inhibits release factor-dependent termination of protein synthesis[28,29]; 2,6-diaminopurine (2,6-DAP), which makes it possible for the tryptophan tRNA to recognise the UGA stop codon[30,31]; and Amlexanox (AMX), which not only promotes PTC-readthrough, but also inhibits the degradation of PTC-bearing mRNA by NMD[32]. The fact that PTCs are ~10-fold more susceptible to translational readthrough than natural stop codons provides a therapeutic window for the use of these molecules as a potential treatment for genetic diseases caused by nonsense mutations[33]. Although PTC suppression efficiency is typically low, coagulation factor deficiencies represent ideal targets for this therapeutic approach, because even minimal increases in factor expression can significantly improve the

bleeding phenotype[34-37]. In this study we have explored the feasibility of readthrough-based therapy for FV deficiency caused by a *F5* nonsense mutation (p.Arg1161Ter) identified in several unrelated patients[38-42] (Table 1). To this end, we have determined residual *F5* mRNA and protein expression in a patient homozygous for *F5* p.Arg1161Ter (to assess *in vivo* NMD and natural readthrough of the mutant *F5* mRNA), and tested the effects of five different readthrough agents in *in vitro* and *ex vivo* models of this mutation.

Table 1. Overview of *F5* c.3481C>T (p.Arg1161Ter) mutation carriers

Patient code in reference	Age	Sex	Geographical origin / ethnicity	Zygosity for c.3481C>T	Other *F5* mutation(s)	FV:C (%)	Bleeding symptoms	Reference
Proband A	16 years	F	Southern Italy	Homozygous	FV Leiden (in *cis*)	<1	Bleeding after tooth extraction, posttraumatic haematoma, menorrhagia	van Wijk et al, 2001 [38]
Proband B *	7 years	M	Southern Italy	Homozygous	FV Leiden (in *cis*)	<1	Asymptomatic	van Wijk et al, 2001 [38]
Case 4	36 years	M	China	Homozygous	-	2	Right knee haemarthrosis since the age of 3 Occasional gum or nose bleeding, joint bleeding	Cao et al, 2008 [39]
Patient 19	Not reported	F	Germany	Homozygous	-	1	Haematoma	Delev et al, 2009 [40]
Patient 20	Not reported	F	Germany	Homozygous	-	1	Haematoma	Delevet al, 2009 [40]
P8	Unknown	Unknown	Iran	Homozygous	-	1	Not available	Paraboschi et al, 2020 [42] Prof. R. Asselta, pers. comm.
Patient **	25 years	M	Korea	Compound heterozygous	c.6027_6032del-GAACAG	4	Bleeding after tooth extraction	Song et al, 2009 [41]
P2	Unknown	Unknown	Iran	Compound heterozygous	c.3924_3927del-TCAG	<1	Not available	Paraboschi et al, 2020 [42] Prof. R. Asselta, pers. comm.
P43	Unknown	M	Italy	Heterozygous	-	50	Asymptomatic	Paraboschi et al, 2020 [42] Prof. R. Asselta, pers. comm.
-	Unknown	M	African/ African American	Heterozygous	-	Unknown	Unknown	gnomAD, August 2023
-	Unknown	M	European (non-Finnish)	Heterozygous	-	Unknown	Unknown	gnomAD, August 2023

* This patient and the patient described in the present report are the same person (Prof. P.M. Mannucci, personal communication).
** Proband of a family with inherited FV deficiency. The *F5* c.3481C>T (p.Arg1161Ter) mutation was also present in his monozygotic twin and in one sister [41].

MATERIALS AND METHODS

Patient characterisation

Blood collection and work-up

The study was approved by the local Institutional Review Board (code nr. 242/2020) and conducted according to the Helsinki declaration. Following written informed consent, venous blood was collected in 0.109 M sodium citrate and in Tempus™ Blood RNA tubes from the FV-deficient patient, his parents and a normal control. Citrated blood was centrifuged at 1200 rpm for 15 minutes (to obtain platelet-rich plasma, PRP) and again at 5000 rpm for 15 minutes (to obtain platelet-poor plasma, PPP). The platelet count in PRP was adjusted to 240,000 platelets/μL using autologous PPP. A second sample of citrated blood, collected 8 years later from the same patient and a normal control, was used to prepare *ex-vivo* differentiated megakaryocytes (see below), as well as PRP and PPP. As a negative control for the *ex vivo* experiments, megakaryocytes were also prepared from a previously characterised FV-deficient patient (PD-III, 0.6% FV) who is compound heterozygous for two missense mutations (*F5* p.Trp255Arg and p.Tyr1623Asp)[6,11,43].

Thrombin generation

Thrombin generation in PRP and PPP was measured using the Calibrated Automated Thrombogram (CAT) method, as described[6].

FV levels

FV activity in plasma and activated washed platelets was quantified using a prothrombinase-based assay, as described[6]. FV antigen levels were determined with an in-house ELISA[18].

TFPIα levels

Plasma TFPIα levels were determined using an in-house ELISA[44].

Genetic analysis

Genomic DNA (gDNA) was isolated from buffy coats using the QIAamp® DNA Blood Mini kit (QIAGEN). All exons and splicing junctions of the patient's *F5* gene were amplified and Sanger-sequenced, essentially as previously described[45].

F5 mRNA analysis

Whole blood RNA was isolated using the Tempus™ Spin RNA Isolation kit (Applied Biosystems) and quantified with a NanoDrop 2000. Total RNA was reverse-transcribed with a *F5*-specific primer using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The *F5* region surrounding the nonsense mutation was amplified

using a forward primer located in exon 13 and a reverse primer located in exon 14 (to prevent co-amplification of gDNA), followed by a nested PCR within exon 13 and direct sequencing. Sequencing peaks were quantified using QSVanalyzer[46]. In a parallel experiment, total RNA was reverse-transcribed with random primers and cDNA fragments corresponding to *F5* exons 5-6 and 18-20 were quantified by qPCR on a Roche LightCycler 480 using GAPDH as house-keeping gene. All primer sequences are available on request.

In vitro model

FV expression constructs

The pMT2/V construct, containing the wild-type *F5* cDNA, was used as template for site-directed mutagenesis (QuikChange II XL Site-Directed Mutagenesis kit; Agilent Technologies) to introduce the FV-Arg1161Ter mutation, alone and in combination with FV-Arg534Gln (FV Leiden)[47].

Cell transfection and treatment

COS-1 cells were maintained at 37°C and 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) with low glucose (Biowest), supplemented with 2 mM L-glutamine, 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin. For each experiment, cells were seeded in 6-well plates at ~70% confluency and transiently transfected with 2 µg *F5* cDNA using Lipofectamine™ 2000 (Invitrogen™) in OptiMEM (Gibco™). Transfected cells were treated with 0-500 µM G418 (Sigma-Aldrich), ELX-02 (MedChemExpress), PTC-124 (Cayman Chemicals), 2,6-DAP (Sigma-Aldrich) or AMX (Sigma-Aldrich) in DMEM. After 48 hours, conditioned media were harvested for FV activity measurements.

FV measurements in cell media

Conditioned media diluted 1:40 (for wild-type FV) or 1:5 (for mutant FV) were assayed for FV activity in a prothrombinase-based assay, as described[11]. FV activity was also evaluated by measuring thrombin generation in 80 µL FV-depleted plasma (Siemens Healthcare) reconstituted with 25 µL conditioned medium. Thrombin generation was determined by CAT after initiation of coagulation with 20 pM TF and 30 µM phospholipid vesicles (DOPS/DOPC/DOPE in a molar ratio of 20/60/20) in the presence of 40 µg/mL thermostable inhibitor of contact activation (TICA).

Cell viability assay

COS-1, Huh-7 and HepG2 cells seeded in 96-well plates were treated with 0-500 µM G418, ELX-02, PTC-124, 2,6-DAP or AMX for 48 hours. Cell viability was determined using the CyQUANT™ XTT Cell Viability Assay (Invitrogen™) according to the manufacturer's instructions.

Ex vivo model

Preparation of ex-vivo differentiated megakaryocytes

Haematopoietic progenitor cells were enriched from citrated blood of the *F5* p.Arg1161Ter-homozygous patient and controls, and differentiated to megakaryocytes as described before[17,43], with minor modifications. Briefly, peripheral blood mononuclear cells isolated by Histopaque-1077 (Sigma-Aldrich) density gradient centrifugation were resuspended in serum-free StemSpanTM-ACF medium (StemCell Technologies) supplemented with 2 mM L-glutamine, 1% insulin-transferrin-selenium (Invitrogen), 10 ng/mL Stem Cell Factor, 50 ng/mL thrombopoietin, 10 ng/mL interleukin-3 and 20 ng/mL interleukin-6 (all from PeproTech). Cells were seeded in 24-well plates containing glass coverslips coated with gelatine (Sigma-Aldrich) and maintained in an incubator at 37°C and 5% CO_2 for 4 days before switching to serum-free Iscove's Modified Dulbecco's Medium (IMDM, Euroclone) supplemented with 2 mM L-glutamine, 1% insulin-transferrin-selenium, as well as 50 ng/mL thrombopoietin and 10 ng/mL interleukin-3. This model, which has been extensively characterised earlier[43], produces megakaryocyte-like cells that can both express and internalise FV.

Treatment with readthrough agents

Starting from day 12, readthrough agents were added to the culture medium at every medium change (twice in 7 days), before retrieving the glass coverslips for immunofluorescence analysis. Based on titrations of each readthrough agent in control megakaryocyte cultures, where toxicity was evaluated by visual inspection of the cells under the microscope, the patient's megakaryocytes were treated with 100-500 μM G418, 500-1000 μM ELX-02, 100-250 μM PTC-124, 50-250 μM 2,6-DAP and 50-100 μM AMX, as well as with 0.7% DMSO (vehicle of PTC-124, 2,6-DAP and AMX) as a negative control.

FV uptake experiments

To test whether FV produced by readthrough of the p.Arg1161Ter mutation could be internalised by patient's megakaryocytes, 0.33 nM wild-type FV (positive control) or mutant FV rescued with G418 or 2,6-DAP was added to the culture medium at day 12 and left for 4 days before immunofluorescence analysis. Conditioned media of COS-1 cells expressing the different FV variants were used as a source of FV, after concentration with Amicon® Ultra-4 Centrifugal Filter Units (Millipore) with 50-kDa cut-off filter.

Immunostaining and fluorescence microscopy

Glass coverslips were processed essentially as previously described[17,43]. Briefly, cells were fixed with 2% paraformaldehyde and permeabilized with 0.5% Triton X-100. FV was visualised with a mouse monoclonal antibody against the human FV light chain

(AHV-5108, Haematologic Technologies) and a fluorescein isothiocyanate (FITC)-labelled goat anti-mouse IgG (Sigma-Aldrich) secondary antibody. Cell nuclei were stained with Draq5TM (Abcam). Slides were mounted with Mowiol anti-fade solution (Sigma-Aldrich) and observed under a Leica DMI6000CS fluorescence microscope (Leica Microsystems CMS) using a 20x/0.40 dry objective or a 63x/1.40 oil-immersion objective. Images were acquired with a DFC365FX camera and analysed using the Leica Application Suite (LAS-AF) 3.1.1 software (Leica Microsystems).

RESULTS

Patient characterisation

The patient is a 29-year-old Italian man with undetectable plasma FV (FV:Ag <1%, FV:C <1%) and a moderate bleeding tendency. He first came to clinical attention at the age of 3 years for a traumatic haemorrhage of the lip, but according to his mother he has been suffering from easy bruising since early infancy. His bleeding history includes recurrent knee haemarthroses from the age of 9 years, an episode of rectal bleeding at age 20 and a right thigh muscle haematoma following a sport trauma at age 21. He is treated on demand with fresh-frozen plasma, or with tranexamic acid for minor bleeding episodes. Both blood samples used in this study were collected at least two months after the last plasma transfusion. As we found out during the preparation of this manuscript, this patient has been reported before as a child[38] (Prof. P.M. Mannucci, personal communication).

Both parents have partial FV deficiency (Figure 1A). The patient's father developed deep-vein thrombosis and massive pulmonary embolism at the age of 32 years after a long car drive, followed by multiple episodes of superficial thrombophlebitis despite oral anticoagulation in the therapeutic range. Thrombophilia screening revealed marked activated protein C (APC) resistance in the ProC Global assay (nAPCsr 0.42, normal range ≥0.80), as well as heterozygosity for the *F2* 20210G>A mutation[48]. After taking vitamin K antagonists for 18 years, he is currently on life-long treatment with apixaban (2.5 mg twice daily). The patient's mother is asymptomatic.

Upon *F5* gene sequencing, the patient proved homozygous for the c.3481C>T mutation in exon 13[38-42], which introduces a premature stop codon (TGA) in the B-domain (p.Arg1161Ter) (Figure 1B). Moreover, he was homozygous for the FV Leiden mutation[47], which however was not expressed due to linkage with the nonsense mutation on both alleles. Accordingly, the patient's parents were heterozygous for both mutations and shared the same *F5* haplotype, suggesting identity by descent. In addition, the patient's father carried the Met2148Thr variant (rs9332701) on the other

F5 allele. This variant has been associated with mildly decreased FV levels[49] and may explain the father's lower FV levels compared to the mother.

Whole blood RNA analysis by qPCR indicated that the expression of *F5* mRNA was markedly reduced in the patient compared to a normal control (relative expression 0.21 and 0.38 for *F5* exons 5-6 and 18-20, respectively), whereas both parents had intermediate levels (Figure 1C). This suggested that the PTC-containing mRNA may be subject to NMD degradation, which was confirmed by direct sequencing of the region surrounding the nonsense mutation in the heterozygous parents (Figure 1B, bottom). However, the mutant *F5* mRNA was only partially degraded and the homozygous patient showed ~30% residual *F5* mRNA (Figure 1B,C).

To assess the patient's overall haemostatic capacity, thrombin generation was measured in PPP and PRP. Thrombin generation in PPP (Figure 2A) was triggered with 1-50 pM TF and 30 μM phospholipids. Control PPP showed progressively shorter lag times and higher peaks at increasing TF concentrations, whereas the patient's PPP did not generate thrombin at any TF concentration, in line with his undetectable plasma FV. Thrombin generation in the mother's PPP (50% FV) was similar to that of control PPP, whereas thrombin generation in the father's PPP (38% FV) was somewhat delayed and decreased, reflecting his ongoing anticoagulant treatment with (at that time) vitamin K antagonists.

Thrombin generation in PRP, initiated with 1-10 pM TF and 20 μg/mL collagen, showed similar trends (Figure 2B). However, thrombin generation in the patient's PRP was measurable, albeit markedly delayed and decreased compared to that of control PRP (lag time 6 min vs. 2 min, peak height 19 nM vs. 104 nM at 10 pM TF). This indicated the presence of residual FV in the patient's platelets, which was confirmed by thrombin generation measurements in an independent plasma sample collected 8 years later (Supplementary Figure S1) as well as by direct measurement of the patient's platelet FV level (3.5% of the normal control).

Plasma TFPIα levels were decreased in the patient (37% and 40% in the two plasma samples) and, to a lesser extent, in his parents (father 74%, mother 51%). Since the *F5* Arg1161Ter mutation is eliminated from the FV-short mRNA by alternative splicing and should therefore not affect the expression of FV-short, the patient's low TFPIα level suggests that not only FV-short but also full-length FV may contribute to stabilising TFPIα in the circulation, in line with earlier TFPIα immunoprecipitation experiments where both FV isoforms were retrieved in the immunoprecipitate[12].

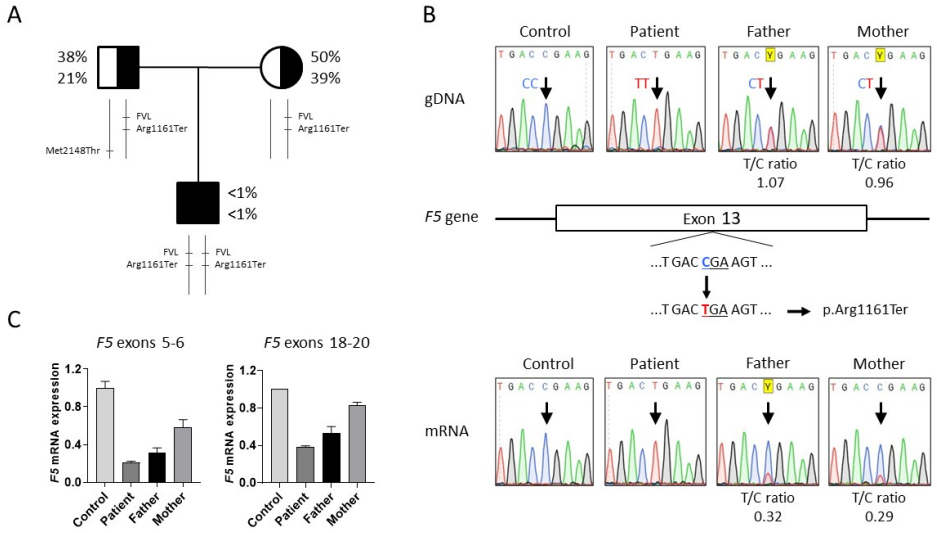


Figure 1. Genetic analysis of the patient and his parents. (A) Pedigree of the affected family. (Half) filled symbols indicate (partial) FV deficiency. FV activity and antigen levels are indicated next to each family member. The bars below each family member represent the two *F5* alleles with the respective genetic variants (FVL, FV Leiden). **(B)** Sequencing chromatograms of the portion of *F5* exon 13 containing the c.3481C>T mutation in the gDNA (top) and mRNA/cDNA (bottom) of the patient, his parents and a normal control. In both parents, that are heterozygous for the *F5* c.3481C>T mutation, the ratio between the peaks corresponding to the mutant (T) and wild-type (C) alleles is close to 1 at the gDNA level, but markedly reduced at the mRNA level, indicating that ~70% of the mutant *F5* mRNA is degraded by NMD. The *F5* c.3481C>T mutation introduces a TGA premature stop codon (p.Arg1161Ter). **(C)** Quantification of *F5* mRNA by RT-qPCR of *F5* exons 5-6 and 18-20 from whole blood RNA of the patient and his parents relative to a normal control.

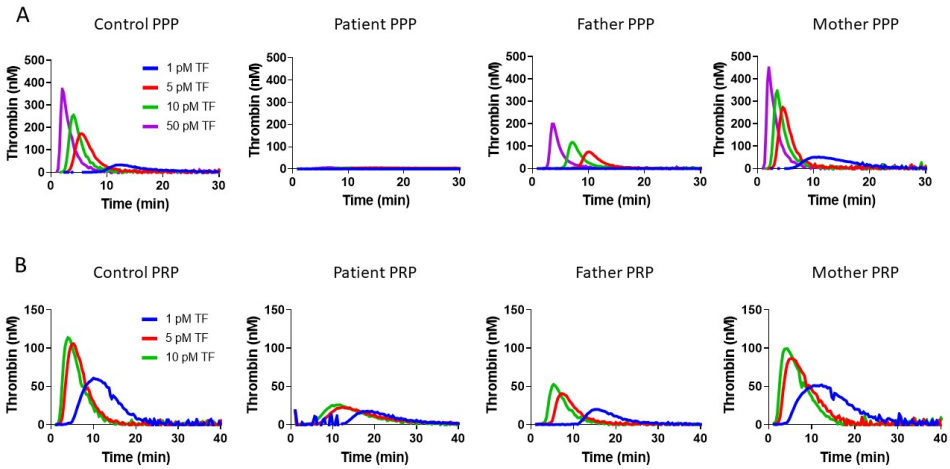


Figure 2. Plasma phenotyping of the patient and his parents. Thrombin generation was measured in platelet-poor plasma (PPP) **(A)** and platelet-rich plasma (PRP) **(B)** of the *F5* p.Arg1161Ter-homozygous patient, his parents and a normal control. Thrombin generation in PPP was initiated with 1-50 pM TF and 30 µM phospholipid vesicles; thrombin generation in PRP was initiated with 1-10 pM TF and 20 µg/mL collagen.

Effects of readthrough agents in an *in vitro* model of *F5* p.Arg1161Ter

COS-1 cells transfected with the FV-Arg1161Ter cDNA were treated with increasing concentrations (0-500 μM) of five different readthrough agents and FV activity in conditioned media was determined using a prothrombinase-based assay. No FV activity was detectable in the media of non-transfected cells, whereas the mutant construct expressed 0.73±0.05% FV activity of the wild-type construct (n=5), mimicking the patient's FV deficiency. Treatment with G418, ELX-02 and 2,6-DAP resulted in a dose-dependent increase of secreted FV activity, whereas PTC-124 and AMX were ineffective (Figure 3A). Compared to untreated cells, G418 increased FV activity up to 7.0±0.9 times, ELX-02 up to 3.1±0.6 times and 2,6-DAP up to 10.8±2.4 times at the highest treatment concentration. These effects could be attributed to the translational readthrough of the mutant *F5* mRNA, because treatment of non-transfected COS-1 cells with 500 μM of each readthrough compound did not result in any detectable prothrombinase activity in the conditioned media, excluding that these molecules stimulate the secretion of FV from the cells or express FVa-like activity themselves.

To check whether the FV produced by translational readthrough of the mutant *F5* mRNA would also function in a more physiological plasma setting, thrombin generation was measured in FV-depleted plasma reconstituted with the conditioned media of untreated and treated cells (Figure 3B). Untreated FV-Arg1161Ter media supported only minimal thrombin generation (peak height 3.1±0.4 nM, n=5) compared to FV-WT media (peak height 277 nM). However, following treatment with increasing concentrations of G418, ELX-02 or 2,6-DAP, FV-Arg1161Ter media supported progressively higher thrombin generation, with maximal peak heights of 22.2 nM (G418), 8.2 nM (ELX-02) and 36.2 nM (2,6-DAP), respectively. Differently, no increase in thrombin generation was observed with media of cells treated with PTC-124 or AMX.

Since AMX mainly targets NMD, whereas G418 and PTC-124 have primarily readthrough activity, cells transfected with FV-Arg1161Ter were also treated with combinations of AMX and G418 or PTC-124. However, no synergistic effects were observed between these compounds (Supplementary Figure S2).

To verify that the FV Leiden mutation does not interfere with PTC readthrough at codon 1161, we compared FV expression by COS-1 cells transfected with FV-Arg1161Ter (single mutant) or with FV-Arg1161Ter in combination with FV Leiden (double mutant) before and after treatment with G418, 2,6-DAP or ELX-02. The single and double mutants expressed comparable FV activity in the media of untreated cells and achieved similar levels of correction after treatment (Supplementary Figure S3).

The effects of increasing concentrations (0-500 μM) of each readthrough agent on cell viability was evaluated in COS-1 cells (used for the *in vitro* model) as well as in the liver cell lines Huh-7 and HepG2 (hepatocytes being the natural site of FV biosynthesis). While ELX-02 was extremely well tolerated up to the highest concentration, all other compounds showed variable degrees of cytotoxicity in the different cell types (Figure 4).

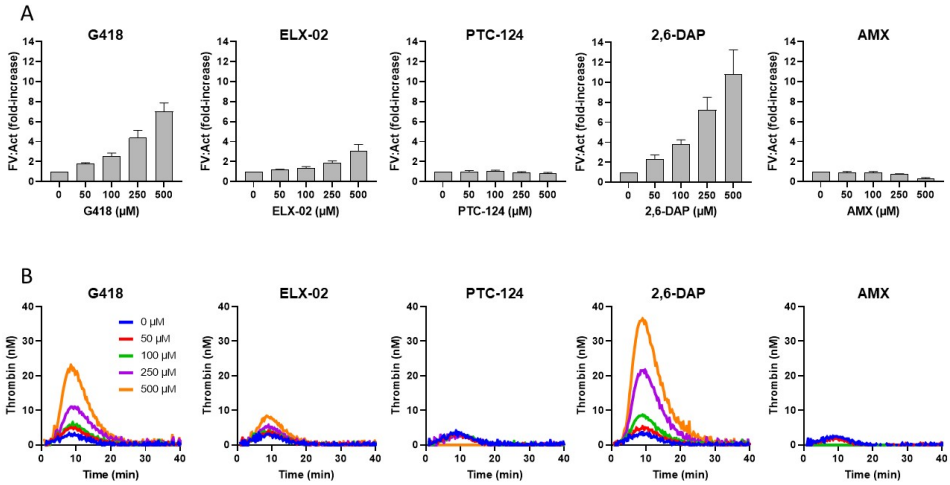


Figure 3. *In vitro* rescue of the FV p.Arg1161Ter mutation. COS-1 cells were transiently transfected with FV-Arg1161Ter cDNA and treated with 0-500 μM G418, ELX-02, PTC-124, 2,6-DAP or AMX for 48 hours. **(A)** FV activity in conditioned media was measured with a prothrombinase-based assay, normalised to the basal FV level in the media of untreated cells, and plotted as a function of treatment concentration. Each bar represents the mean and standard deviation of three biological replicates. **(B)** Thrombin generation in FV-depleted plasma reconstituted with conditioned media of untreated and treated cells. Thrombin generation was initiated with 20 pM tissue factor and 30 μM phospholipid vesicles.

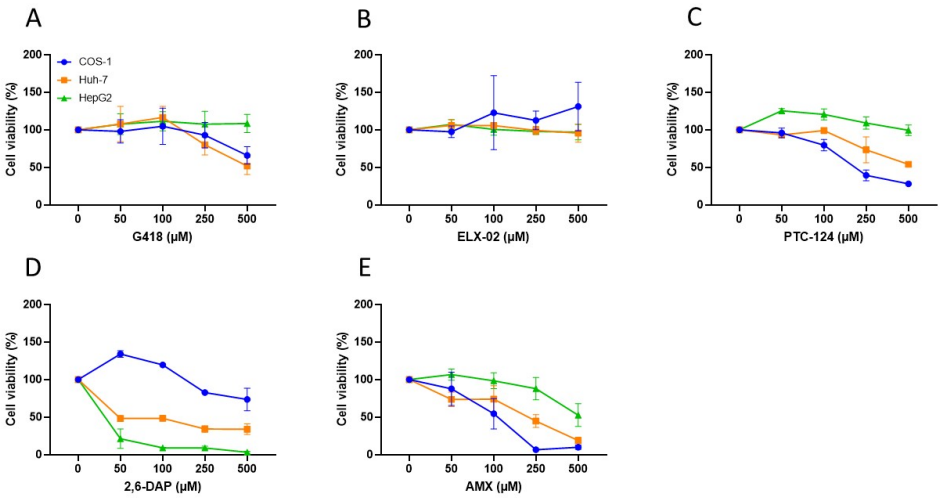


Figure 4. *In vitro* cytotoxicity of the readthrough compounds. COS-1, Huh-7 and HepG2 cells were treated with 0-500 µM G418, ELX-02, PTC-124, 2,6-DAP or AMX for 48 hours and cell viability was assessed with an XTT-based assay. Results are expressed as percentage of the basal cell viability in the absence of treatment. Each bar represents the mean and standard deviation of three biological replicates (two for ELX-02).

Effects of readthrough agents in an *ex vivo* model of *F5* p.Arg1161Ter

The rescuing efficacy of the different readthrough agents was also tested in *ex-vivo* differentiated megakaryocytes[17,43] of the *F5* p.Arg1161Ter-homozygous patient. To this end, haematopoietic progenitor cells were cultured in the presence of thrombopoietin and interleukin-3 for 11 days to promote megakaryocyte differentiation, treated with readthrough agents (or not) for the next 7 days, and analysed by immunofluorescence. Treatment concentrations were adjusted according to preliminary titrations of each readthrough agent in control megakaryocyte cultures to determine the maximum concentration that could be tolerated without excessive toxicity. FV was visualised using a monoclonal antibody directed against the light chain (i.e. the C-terminal portion) of FV, to ensure exclusive detection of the full-length readthrough product. Based on this immunofluorescence staining, megakaryocytes from a normal control showed abundant intracellular expression of FV (Figure 5A), whereas no FV could be detected in the untreated patient's megakaryocytes (Figure 5B). All five readthrough agents restored FV expression in the patient's megakaryocytes (Figure 5C-G, Supplementary Figure S4A-G), whereas 0.7% DMSO (vehicle) was completely ineffective (Supplementary Figure S4B). As a control, the same treatments were performed on megakaryocytes of a different FV-deficient patient (PD-III)[6,11,43] who is compound heterozygous for missense mutations and should therefore not respond to readthrough agents. As expected, no rescue of FV expression was observed in this

case (Supplementary Figure S5), confirming the specificity of the readthrough agents for nonsense mutations and excluding that they induce aspecific green fluorescence in the treated cells.

When untreated patient's megakaryocytes were cultured in the presence of FV variants produced in COS-1 cells, they proved able to internalise not only wild-type FV, but also FV obtained by rescuing the *F5* p.Arg1161Ter mutation with G418 (which mostly incorporates the natural amino acid Arg at the PTC site)[50] or 2,6-DAP (which incorporates Trp[30]) (Figure 6, Supplementary Figure S6). The same was true for megakaryocytes of patient PD-III (Supplementary Figure S7). This indicates that the positive FV immunostaining truly reflects FV internalisation rather than endogenous FV expression induced by traces of readthrough agents present in the concentrated media that were used as a source of G418- or 2,6-DAP-rescued mutant FV.

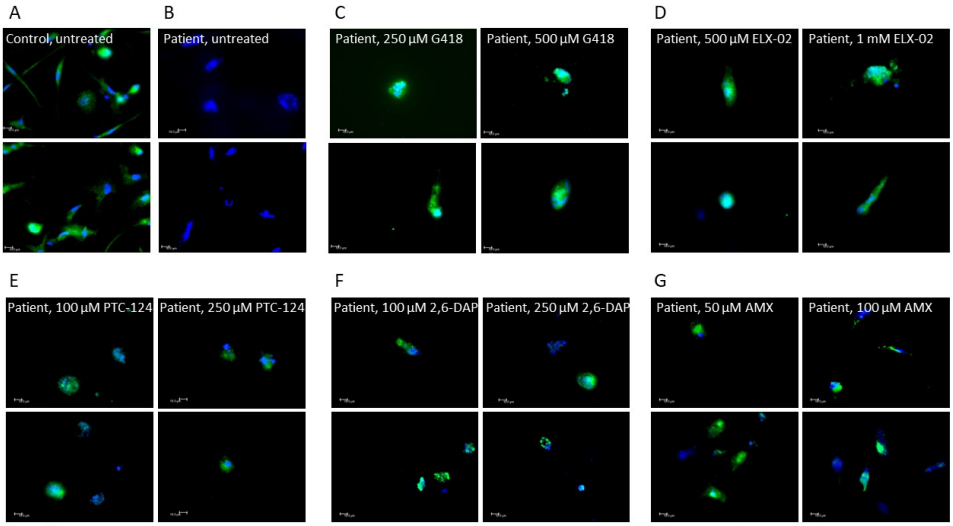


Figure 5. Effect of readthrough agents on FV expression in *ex-vivo* differentiated megakaryocytes of the *F5* p.Arg1161Ter-homozygous patient. Haematopoietic progenitor cells isolated from peripheral blood of a normal control and the *F5* p.Arg1161Ter-homozygous patient were cultured in the presence of thrombopoietin and interleukin-3 to induce megakaryocyte differentiation. Starting from day 12, cells were treated with readthrough compounds for 7 days and then processed for immunofluorescence analysis. Cell nuclei were stained with Draq5TM (blue). FV was visualised using a mouse monoclonal antibody against the light chain of human FV (AHV-5108), which recognises only full-length FV molecules, followed by a FITC-labelled goat anti-mouse IgG antibody (green). Glass slides were observed under a Leica DMI6000CS fluorescence microscope using a 63x/1.40 oil-immersion objective. Images (overlay of blue and green fluorescence channels) were acquired with a DFC365FX camera and analysed using the LAS-AF 3.1.1 software. The different panels show two representative microscope fields for each of the following conditions: untreated control megakaryocytes **(A)**, untreated patient's megakaryocytes **(B)** and patient's megakaryocytes treated with the indicated concentrations of G418 **(C)**, ELX-02 **(D)**, PTC-124 **(E)**, 2,6-DAP **(F)** and AMX **(G)**. Scale bar: 10 µm.

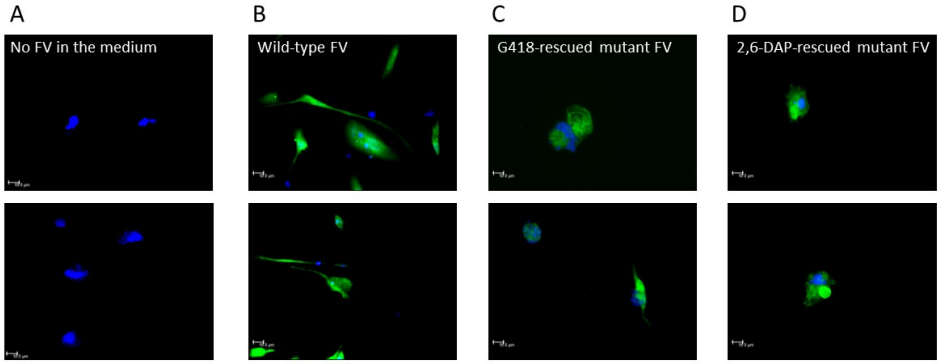


Figure 6. Uptake of rescued mutant FV by *ex-vivo* differentiated megakaryocytes of the *F5* p.Arg1161Ter-homozygous patient. Haematopoietic progenitor cells isolated from peripheral blood of the *F5* p.Arg1161Ter-homozygous patient were cultured in the presence of thrombopoietin and interleukin-3 to induce megakaryocyte differentiation. Starting from day 12, cells were either left unexposed **(A)** or exposed for 4 days to 0.33 nM wild-type FV **(B)**, 0.33 nM mutant FV rescued with G418 **(C)**, or 0.33 nM mutant FV rescued with 2,6-DAP **(D)** in the culture medium, before being processed for immunofluorescence analysis. Cell nuclei were stained with Draq5TM (blue). Internalised FV was visualised using a mouse monoclonal antibody against the light chain of human FV (AHV-5108), followed by a FITC-labelled goat anti-mouse IgG antibody (green). Glass slides were observed under a Leica DMI6000CS fluorescence microscope using a 63x/1.40 oil-immersion objective. Images (overlay of blue and green fluorescence channels) were acquired with a DFC365FX camera and analysed using the LAS-AF 3.1.1 software. Two representative microscope fields are shown for each condition. Scale bar: 10 µm.

DISCUSSION

In contrast to all other coagulation factor deficiencies, for which concentrates and/or recombinant preparations of the missing factor are available, FV-deficient patients are still dependent on fresh-frozen plasma for their treatment and prophylaxis. Although effective, (repeated) plasma administration is burdensome and not without risks[14], calling for alternative therapeutic strategies for FV deficiency[15-17,19]. In this study, we show that the *F5* p.Arg1161Ter nonsense mutation is amenable to pharmacological readthrough in *in vitro* and *ex vivo* models. To our knowledge, this is the first study on readthrough therapy of FV deficiency.

While most *F5* mutations are private, the *F5* p.Arg1161Ter mutation has been reported in at least nine unrelated FV-deficient patients of Caucasian or Asian descent[38-42] and in two subjects of the gnomAD database (https://gnomad.broadinstitute.org, accessed in August 2023), one of whom of African ancestry (Table 1). Its prevalence and widespread geographical distribution suggest that this C>T transition at a CpG dinucleotide has occurred independently more than once.

The outcome of a nonsense mutation depends on the extent of mRNA degradation by NMD and on the susceptibility of the PTC to translational readthrough. As observed earlier[38] and confirmed here, the *F5* p.Arg1161Ter mRNA is only partially degraded by NMD, possibly because the PTC is located in the middle of an extremely large exon (2821 bp), which decreases NMD efficiency[51]. Moreover, the stop codon introduced by the p.Arg1161Ter mutation (UGA) is the most permissive to translational readthrough, even if followed by a less favourable A[24,52]. Accordingly, the absence of life-threatening bleeding manifestations in all *F5* p.Arg1161Ter homozygotes described so far (Table 1) indicates that this nonsense mutation allows some full-length FV expression by low-frequency natural readthrough of the PTC. While quickly cleared from plasma, these FV traces accumulate in the platelet α-granules (as demonstrated by the measurable platelet FV and thrombin generation in our patient's PRP) and can support minimal haemostasis[6,53], especially in combination with low plasma TFPIα levels[11].

These favourable characteristics predicted that the *F5* p.Arg1161Ter mutation could respond to treatment with molecules that enhance translational readthrough and/or suppress NMD. To verify this, we screened five readthrough agents with different mechanisms of action in COS-1 cells transfected with FV-Arg1161Ter cDNA (*in vitro* model) and in *ex-vivo* differentiated megakaryocytes of a *F5* p.Arg1161Ter-homozygous patient (*ex vivo* model).

The *in vitro* model allowed quantitative assessment of the efficacy and cytotoxicity of the different readthrough agents, revealing three active compounds. G418 increased secreted FV activity up to 7 times, in line with similar studies on other coagulations factor deficiencies[34-36]. Unfortunately, the clinical use of this compound is limited by its oto- and nephrotoxicity[54,55]. The synthetic aminoglycoside analogue ELX-02, which is currently in phase 2 clinical trials for other genetic diseases [26,27], was somewhat less effective, increasing FV activity up to ~3 times. Interestingly, ELX-02 did not show any in vitro cytotoxicity up to a concentration of 500 μM, which is in line with its promising *in vivo* safety profile[26]. The most potent compound was 2,6-DAP, which increased FV activity up to ~11 times, but also showed considerable toxicity in two liver cell lines. However, no toxicity was observed in other studies, including *in vivo* mouse models[30,31].

The FV produced by readthrough of the p.Arg1161Ter mutation was fully functional not only in a prothrombinase assay with purified components, but also in a plasma-based thrombin generation assay. This indicates that the replacement of Arg1161 by a different amino acid as a result of the readthrough process is structurally and functionally well tolerated, consistent with the B-domain being largely unstructured and removed upon FV activation. This may also explain the remarkable density of nonsense mutations in the B-domain of FV compared to other domains[4], because natural readthrough of B-domain

PTCs would be more likely to result in a viable protein, as recently proposed for the B-domain of factor VIII[56]. Accordingly, other nonsense mutations in the FV B-domain might also respond to pharmacological induction of translational readthrough, potentially extending the relevance of our study to a larger group of FV-deficient patients. However, each mutation should be evaluated individually[37], as readthrough success critically depends on the type of stop codon and its sequence context[24].

PTC-124 and AMX (alone or in combination) did not rescue the p.Arg1161Ter mutation *in vitro*, and even decreased FV activity at the highest treatment concentrations, probably due to progressive cell death. In the case of AMX, which is primarily an NMD inhibitor[32], the observed toxicity may reflect the importance of NMD as a physiological protection mechanism against truncated and potentially harmful proteins[51]. More in general, the toxicity of readthrough agents may derive from imperfect discrimination between PTCs and natural stop codons. Accordingly, major research efforts are ongoing to identify new active compounds with reduced toxicity[57-60].

Although megakaryocytes are not the natural source of FV *in vivo*[7,8], they can synthesise FV in culture[43,61,62], providing a model to test the readthrough agents directly on patients' cells *ex vivo*. Interestingly, all five readthrough agents effectively restored FV expression in cultured megakaryocytes of our *F5* p.Arg1161Ter-homozygous patient, as qualitatively assessed by immunofluorescence staining. Despite the absence of a clear dose-response, the specificity of the observed effects is supported by 1) the use of a monoclonal antibody against the FV light chain (recognising only the full-length readthrough product); 2) the ability of the readthrough agents to restore FV expression in megakaryocytes of the *F5* p.Arg1161Ter-homozygous patient but not in those of a patient with missense mutations; and 3) the lack of effect of 0.7% DMSO (vehicle). Unfortunately, due to the low number of cells and technical difficulties, we were unable to quantify FV expression in treated megakaryocyte lysates by ELISA and/or functional assays. The discrepancy between the *in vitro* model, where PTC-124 and AMX did not rescue mutant FV expression, and the *ex vivo* model, where all readthrough agents were effective, might be explained, at least in part, by the absence of introns in the *F5* cDNA used to transfect COS-1 cells. In fact, NMD (the main target of AMX[32]) and premature termination of translation (targeted by PTC-124[29]) are inter-dependent[52] and tightly coupled to pre-mRNA splicing[21], which is bypassed in the absence of introns. In this respect, the *ex vivo* model is much more physiological than the *in vitro* model and could be further improved by using induced pluripotent stem cell (iPSC)-derived patient's hepatocytes, which are the natural site of FV biosynthesis and would therefore optimally mimic the transcriptional and post-transcriptional regulation of *F5* gene expression as it occurs *in vivo*. The use of physiologically relevant test models has been recently emphasised as a key factor to bridge the translational gap of readthrough agents[63].

For readthrough-based therapy of FV deficiency to be successful *in vivo*, the little FV produced by PTC readthrough in hepatocytes and secreted in plasma should be internalised by bone-marrow megakaryocytes, to build and maintain the protective platelet FV pool[6,53]. The ability of *ex-vivo* differentiated megakaryocytes to endocytose exogenous FV[43] allowed us to verify that mutant FV rescued with G418 and 2,6-DAP can be efficiently internalised, even at the very low concentration of 0.33 nM, corresponding to a plasma FV level of ~1.5%. This is consistent with FV uptake by megakaryocytes being mediated by the light chain, without involvement of the B-domain[64].

The FV Leiden mutation[47], present in our patient but absent in most *F5* p.Arg1161Ter carriers, did not interfere with readthrough-mediated rescue of the p.Arg1161Ter mutation *in vitro*. *In vivo*, the procoagulant properties of FV Leiden would help the patient to achieve adequate haemostasis at very low FV, but expression of slightly higher levels of FV Leiden in the setting of low TFPIα levels (37-40%) could expose him to a risk of thrombosis, as recently observed in a patient with very low levels (3%) of another APC-resistant FV variant (FV Besançon)[65]. This may be even more relevant to our patient in view of his family history of thrombosis. In fact, although his father's thrombotic events may be ascribed to a combination of high prothrombin (due to the F2 20210G>A mutation) and decreased FV and TFPIα levels, all of which are known to contribute to APC resistance[66], it cannot be excluded that an unknown thrombophilic defect segregates in the family.

In conclusion, we have obtained *in vitro* and *ex vivo* proof-of-principle for readthrough-mediated rescue of functional FV from the *F5* p.Arg1161Ter nonsense mutation. The extent to which this approach could be applicable to other F5 nonsense mutations, particularly outside of the B domain, remains to be determined. Moreover, the *in vivo* efficacy as well as the short- and long-term safety of this form of therapy need to be addressed in future studies.

REFERENCES

1. Dahlbäck, B., *Pro- and anticoagulant properties of factor V in pathogenesis of thrombosis and bleeding disorders.* Int J Lab Hematol, 2016. **38 Suppl 1**: p. 4-11.
2. Rosing, J., et al., *The role of phospholipids and factor Va in the prothrombinase complex.* J Biol Chem, 1980. **255**(1): p. 274-83.
3. Cui, J., et al., *Fatal haemorrhage and incomplete block to embryogenesis in mice lacking coagulation factor V.* Nature, 1996. **384**(6604): p. 66-8.
4. Tabibian, S., et al., *A Comprehensive Overview of Coagulation Factor V and Congenital Factor V Deficiency.* Semin Thromb Hemost, 2019. **45**(5): p. 523-543.
5. Lak, M., et al., *Symptoms of inherited factor V deficiency in 35 Iranian patients.* Br J Haematol, 1998. **103**(4): p. 1067-9.
6. Duckers, C., et al., *Residual platelet factor V ensures thrombin generation in patients with severe congenital factor V deficiency and mild bleeding symptoms.* Blood, 2010. **115**(4): p. 879-86.
7. Camire, R.M., et al., *Secretable human platelet-derived factor V originates from the plasma pool.* Blood, 1998. **92**(9): p. 3035-41.
8. Gould, W.R., et al., *Megakaryocytes endocytose and subsequently modify human factor V in vivo to form the entire pool of a unique platelet-derived cofactor.* J Thromb Haemost, 2005. **3**(3): p. 450-6.
9. van Doorn, P., et al., *The C-terminus of tissue factor pathway inhibitor-alpha inhibits factor V activation by protecting the Arg(1545) cleavage site.* J Thromb Haemost, 2017. **15**(1): p. 140-149.
10. Wood, J.P., et al., *Tissue factor pathway inhibitor-alpha inhibits prothrombinase during the initiation of blood coagulation.* Proc Natl Acad Sci U S A, 2013. **110**(44): p. 17838-43.
11. Duckers, C., et al., *Low plasma levels of tissue factor pathway inhibitor in patients with congenital factor V deficiency.* Blood, 2008. **112**(9): p. 3615-23.
12. Vincent, L.M., et al., *Coagulation factor V(A2440G) causes east Texas bleeding disorder via TFPIalpha.* J Clin Invest, 2013. **123**(9): p. 3777-87.
13. Bulato, C., et al., *"In vitro" correction of the severe factor V deficiency-related coagulopathy by a novel plasma-derived factor V concentrate.* Haemophilia, 2018. **24**(4): p. 648-656.
14. Pandey, S. and G.N. Vyas, *Adverse effects of plasma transfusion.* Transfusion, 2012. **52 Suppl 1**: p. 65S-79S.
15. DesPain, A.W., et al., *Management of intracranial hemorrhage in severe factor V deficiency and definitive treatment with liver transplantation.* Pediatr Transplant, 2018. **22**(1).
16. Serrano, L.J., et al., *Cell therapy for factor V deficiency: An approach based on human decidua mesenchymal stem cells.* Biomed Pharmacother, 2021. **142**: p. 112059.
17. Nuzzo, F., et al., *Antisense-based RNA therapy of factor V deficiency: in vitro and ex vivo rescue of a F5 deep-intronic splicing mutation.* Blood, 2013. **122**(23): p. 3825-31.
18. Nuzzo, F., et al., *Characterization of an apparently synonymous F5 mutation causing aberrant splicing and factor V deficiency.* Haemophilia, 2015. **21**(2): p. 241-248.
19. Nakamura, T., et al., *Successful correction of factor V deficiency of patient-derived iPSCs by CRISPR/Cas9-mediated gene editing.* Haemophilia, 2020. **26**(5): p. 826-833.
20. Efthymiou, C., et al., *Analysis of 363 Genetic Variants in F5 via an Interactive Web Database Reveals New Insights into FV Deficiency and FV Leiden.* TH Open, 2023. **7**(1): p. e30-e41.
21. Holbrook, J.A., et al., *Nonsense-mediated decay approaches the clinic.* Nat Genet, 2004. **36**(8): p. 801-8.
22. Blanchet, S., et al., *New insights into the incorporation of natural suppressor tRNAs at stop codons in Saccharomyces cerevisiae.* Nucleic Acids Res, 2014. **42**(15): p. 10061-72.
23. Keeling, K.M. and D.M. Bedwell, *Suppression of nonsense mutations as a therapeutic approach to treat genetic diseases.* Wiley Interdiscip Rev RNA, 2011. **2**(6): p. 837-52.

24. Lombardi, S., et al., *Molecular Insights into Determinants of Translational Readthrough and Implications for Nonsense Suppression Approaches.* Int J Mol Sci, 2020. **21**(24): p. 9449.

25. Manuvakhova, M., K. Keeling, and D.M. Bedwell, *Aminoglycoside antibiotics mediate context-dependent suppression of termination codons in a mammalian translation system.* RNA, 2000. **6**(7): p. 1044-55.

26. Kerem, E., *ELX-02: an investigational read-through agent for the treatment of nonsense mutation-related genetic disease.* Expert Opin Investig Drugs, 2020. **29**(12): p. 1347-1354.

27. Crawford, D.K., et al., *ELX-02 Generates Protein via Premature Stop Codon Read-Through without Inducing Native Stop Codon Read-Through Proteins.* J Pharmacol Exp Ther, 2020. **374**(2): p. 264-272.

28. Welch, E.M., et al., *PTC124 targets genetic disorders caused by nonsense mutations.* Nature, 2007. **447**(7140): p. 87-91.

29. Ng, M.Y., et al., *Ataluren and aminoglycosides stimulate read-through of nonsense codons by orthogonal mechanisms.* Proc Natl Acad Sci U S A, 2021. **118**(2): p. e2020599118.

30. Trzaska, C., et al., *2,6-Diaminopurine as a highly potent corrector of UGA nonsense mutations.* Nat Commun, 2020. **11**(1): p. 1509.

31. Leroy, C., et al., *Use of 2,6-diaminopurine as a potent suppressor of UGA premature stop codons in cystic fibrosis.* Mol Ther, 2023. **31**(4): p. 970-985.

32. Gonzalez-Hilarion, S., et al., *Rescue of nonsense mutations by amlexanox in human cells.* Orphanet J Rare Dis, 2012. **7**: p. 58.

33. Bidou, L., et al., *Sense from nonsense: therapies for premature stop codon diseases.* Trends Mol Med, 2012. **18**(11): p. 679-88.

34. Pinotti, M., et al., *Intracellular readthrough of nonsense mutations by aminoglycosides in coagulation factor VII.* J Thromb Haemost, 2006. **4**(6): p. 1308-14.

35. Branchini, A., et al., *Differential functional readthrough over homozygous nonsense mutations contributes to the bleeding phenotype in coagulation factor VII deficiency.* J Thromb Haemost, 2016. **14**(10): p. 1994-2000.

36. Ferrarese, M., et al., *Secretion of wild-type factor IX upon readthrough over F9 pre-peptide nonsense mutations causing hemophilia B.* Hum Mutat, 2018. **39**(5): p. 702-708.

37. Martorell, L., et al., *Variable readthrough responsiveness of nonsense mutations in hemophilia A.* Haematologica, 2020. **105**(2): p. 508-518.

38. van Wijk, R., et al., *Coexistence of a novel homozygous nonsense mutation in exon 13 of the factor V gene with the homozygous Leiden mutation in two unrelated patients with severe factor V deficiency.* Br J Haematol, 2001. **114**(4): p. 871-4.

39. Cao, L.J., et al., *[Gene analysis of five inherited factor V deficiency cases].* Zhonghua Xue Ye Xue Za Zhi, 2008. **29**(3): p. 145-8.

40. Delev, D., et al., *Factor 5 mutation profile in German patients with homozygous and heterozygous factor V deficiency.* Haemophilia, 2009. **15**(5): p. 1143-53.

41. Song, J., et al., *A novel in-frame deletion in the factor V C1 domain associated with severe coagulation factor V deficiency in a Korean family.* Blood Coagul Fibrinolysis, 2009. **20**(2): p. 150-6.

42. Paraboschi, E.M., et al., *Profiling the mutational landscape of coagulation factor V deficiency.* Haematologica, 2020. **105**(4): p. e180-e185.

43. Radu, C.M., et al., *Endocytosis of exogenous factor V by ex-vivo differentiated megakaryocytes from patients with severe parahaemophilia.* Br J Haematol, 2016. **175**(3): p. 517-524.

44. Maurissen, L.F., et al., *Thrombin generation-based assays to measure the activity of the TFPI-protein S pathway in plasma from normal and protein S-deficient individuals.* J Thromb Haemost, 2010. **8**(4): p. 750-8.

45. Castoldi, E., et al., *Homozygous F5 deep-intronic splicing mutation resulting in severe factor V deficiency and undetectable thrombin generation in platelet-rich plasma.* J Thromb Haemost, 2011. **9**(5): p. 959-68.

46. Carr, I.M., et al., *Inferring relative proportions of DNA variants from sequencing electropherograms.*

Bioinformatics, 2009. **25**(24): p. 3244-50.

47. Bertina, R.M., et al., *Mutation in blood coagulation factor V associated with resistance to activated protein C.* Nature, 1994. **369**(6475): p. 64-7.

48. Poort, S.R., et al., *A common genetic variation in the 3'-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis.* Blood, 1996. **88**(10): p. 3698-703.

49. Scanavini, D., et al., *Modulation of factor V levels in plasma by polymorphisms in the C2 domain.* Arterioscler Thromb Vasc Biol, 2004. **24**(1): p. 200-6.

50. Roy, B., et al., *Ataluren stimulates ribosomal selection of near-cognate tRNAs to promote nonsense suppression.* Proc Natl Acad Sci U S A, 2016. **113**(44): p. 12508-12513.

51. Supek, F., B. Lehner, and R.G.H. Lindeboom, *To NMD or Not To NMD: Nonsense-Mediated mRNA Decay in Cancer and Other Genetic Diseases.* Trends Genet, 2021. **37**(7): p. 657-668.

52. Embree, C.M., R. Abu-Alhasan, and G. Singh, *Features and factors that dictate if terminating ribosomes cause or counteract nonsense-mediated mRNA decay.* J Biol Chem, 2022. **298**(11): p. 102592.

53. Bouchard, B.A., et al., *Platelets and platelet-derived factor Va confer hemostatic competence in complete factor V deficiency.* Blood, 2015. **125**(23): p. 3647-50.

54. Guthrie, O.W., *Aminoglycoside induced ototoxicity.* Toxicology, 2008. **249**(2-3): p. 91-6.

55. Lopez-Novoa, J.M., et al., *New insights into the mechanism of aminoglycoside nephrotoxicity: an integrative point of view.* Kidney Int, 2011. **79**(1): p. 33-45.

56. Testa, M.F., et al., *Translational readthrough at F8 nonsense variants in factor VIII B domain contributes to residual expression and lowers inhibitor association.* Haematologica, 2023. **108**(2): p. 472-482.

57. Benhabiles, H., et al., *Optimized approach for the identification of highly efficient correctors of nonsense mutations in human diseases.* PLoS One, 2017. **12**(11): p. e0187930.

58. Tutone, M., et al., *Pharmacophore-Based Design of New Chemical Scaffolds as Translational Readthrough-Inducing Drugs (TRIDs).* ACS Med Chem Lett, 2020. **11**(5): p. 747-753.

59. Popadynec, M., et al., *Reducing the Toxicity of Designer Aminoglycosides as Nonsense Mutation Readthrough Agents for Therapeutic Targets.* ACS Med Chem Lett, 2021. **12**(9): p. 1486-1492.

60. Sharma, J., et al., *A small molecule that induces translational readthrough of CFTR nonsense mutations by eRF1 depletion.* Nat Commun, 2021. **12**(1): p. 4358.

61. Gewirtz, A.M., et al., *Biology of human megakaryocyte factor V.* Blood, 1986. **67**(6): p. 1639-48.

62. Giampaolo, A., et al., *Factor-V expression in platelets from human megakaryocytic culture.* Br J Haematol, 2005. **128**(1): p. 108-11.

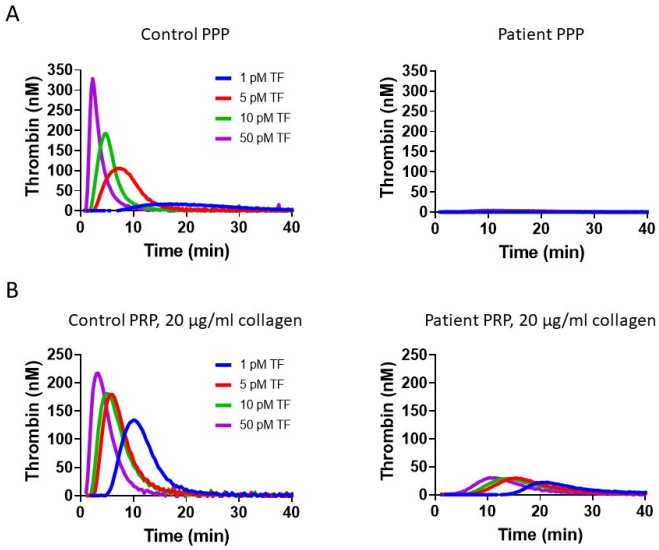
63. Spelier, S., et al., *Readthrough compounds for nonsense mutations: bridging the translational gap.* Trends Mol Med, 2023. **29**(4): p. 297-314.

64. Bouchard, B.A., S. Abdalla, and P.B. Tracy, *The factor V light chain mediates the binding and endocytosis of plasma-derived factor V by megakaryocytes.* J Thromb Haemost, 2013. **11**(12): p. 2181-3.

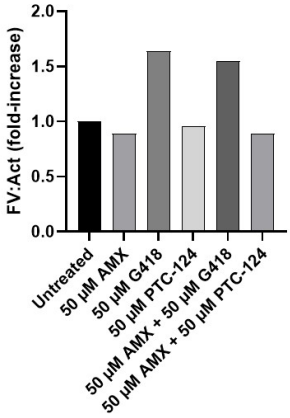
65. Castoldi, E., et al., *Severe thrombophilia in a factor V-deficient patient homozygous for the Ala2086Asp mutation (FV Besançon).* J Thromb Haemost, 2021. **19**(5): p. 1186-1199.

66. de Visser, M.C., et al., *Determinants of the APTT- and ETP-based APC sensitivity tests.* J Thromb Haemost, 2005. **3**(7): p. 1488-94.

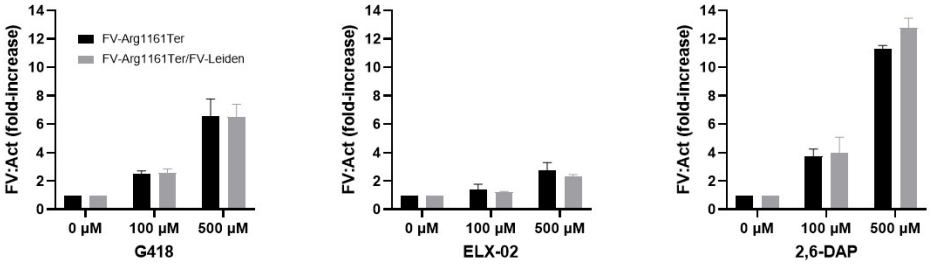
SUPPLEMENTARY MATERIAL



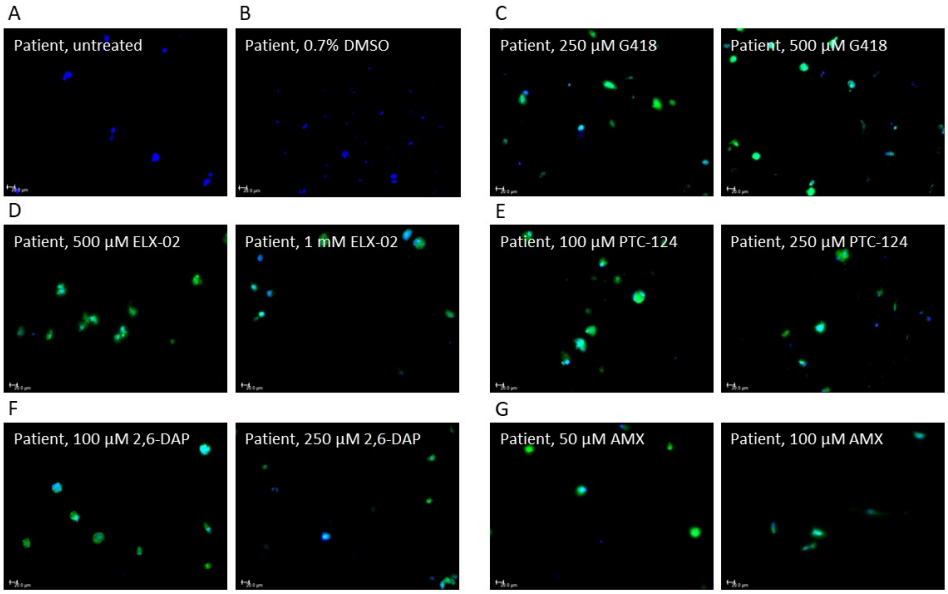
Supplementary Figure S1. Replication of thrombin generation in an independent plasma sample of the *F5* p.Arg1161Ter-homozygous patient. Thrombin generation was measured in platelet-poor plasma (PPP) **(A)** and platelet-rich plasma (PRP) **(B)** of the *F5* p.Arg1161Ter-homozygous patient and a normal control. Thrombin generation in PPP was initiated with 1-50 pM TF and 30 µM phospholipid vesicles; thrombin generation in PRP was initiated with 1-50 pM TF and 20 µg/mL collagen. The blood samples used to prepare PRP and PPP for this experiment were collected 8 years later than those used for the experiment presented in Figure 2. The control subject was different from the one in Figure 2.



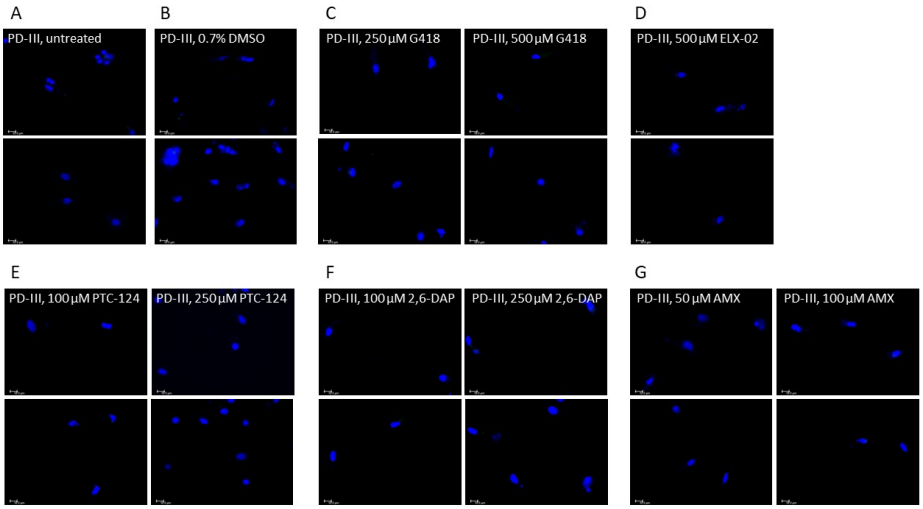
Supplementary Figure S2. *In vitro* rescue of the *F5* p.Arg1161Ter mutation by combinations of readthrough agents. COS-1 cells were transfected with FV-Arg1161Ter cDNA and treated with 50 µM AMX, G418 or PTC-124 alone, or with 50 µM AMX in combination with 50 µM G418 or PTC-124, for 48 hours. FV activity in conditioned media was measured with a prothrombinase-based assay, normalised to the basal FV level in the medium of untreated cells, and plotted as a function of treatment.



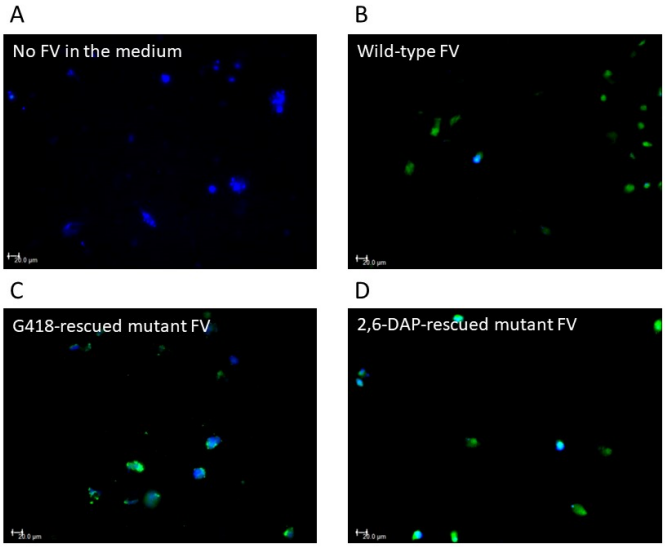
Supplementary Figure S3. Effect of FV Leiden on the *in vitro* rescue of the *F5* p.Arg1161Ter mutation. COS-1 cells were transfected with FV-Arg1161Ter (single mutant) or FV-Arg1161Ter/FV-Leiden (double mutant) cDNA and treated with 0, 100 or 500 µM G418, ELX-02 or 2,6-DAP for 48 hours. FV activity in conditioned media was measured with a prothrombinase-based assay, normalised to the basal FV level in media of untreated cells, and plotted as a function of treatment concentration. Each bar represents the mean and standard deviation of two biological replicates.



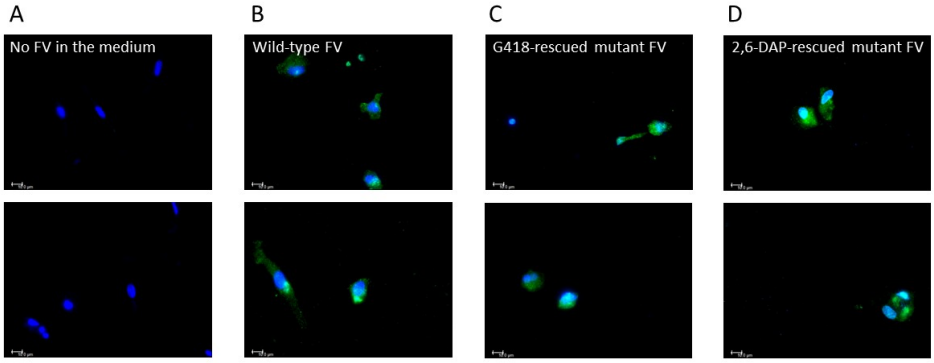
Supplementary Figure S4. Effect of readthrough agents on FV expression in *ex-vivo* differentiated megakaryocytes of the *F5* p.Arg1161Ter-homozygous patient (low-magnification images). Haematopoietic progenitor cells isolated from peripheral blood of the *F5* p.Arg1161Ter-homozygous patient were cultured in the presence of thrombopoietin and interleukin-3 to induce megakaryocyte differentiation. Starting from day 12, cells were treated with readthrough compounds for 7 days and then processed for immunofluorescence analysis. Cell nuclei were stained with Draq5TM (blue). FV was visualised using a mouse monoclonal antibody against the light chain of human FV (AHV-5108), which recognises only full-length FV molecules, followed by a FITC-labelled goat anti-mouse IgG antibody (green). Glass slides were observed under a Leica DMI6000CS fluorescence microscope using a 20x/0.40 dry objective. Images (overlay of blue and green fluorescence channels) were acquired with a DFC365FX camera and analysed using the LAS-AF 3.1.1 software. The different panels show a representative microscope field for each of the following conditions: untreated patient's megakaryocytes **(A)**, patient's megakaryocytes treated with 0.7% DMSO (negative control) **(B)** and patient's megakaryocytes treated with the indicated concentrations of G418 **(C)**, ELX-02 **(D)**, PTC-124 **(E)**, 2,6-DAP **(F)** and AMX **(G)**. Scale bar: 20 µm. These images taken at low magnification convey an impression of the percentage of FV-positive cells after treatment. Cytotoxicity is visible in some panels as a lower number of cells.



Supplementary Figure S5. Effect of readthrough agents on FV expression in *ex-vivo* differentiated megakaryocytes of a FV-deficient patient with missense mutations. Haematopoietic progenitor cells isolated from peripheral blood of FV-deficient patient PD-III, who is compound heterozygous for the *F5* p.Trp255Arg and p.Tyr1623Asp missense mutations[1-3], were cultured in the presence of thrombopoietin and interleukin-3 to induce megakaryocyte differentiation. Starting from day 12, cells were treated with readthrough compounds for 7 days and then processed for immunofluorescence analysis. Cell nuclei were stained with Draq5TM (blue). FV was visualised using a mouse monoclonal antibody against the light chain of human FV (AHV-5108), which recognises only full-length FV molecules, followed by a FITC-labelled goat anti-mouse IgG antibody (green, no FV expression could be detected). Glass slides were observed under a Leica DMI6000CS fluorescence microscope using a 63x/1.40 oil-immersion objective. Images (overlay of blue and green fluorescence channels) were acquired with a DFC365FX camera and analysed using the LAS-AF 3.1.1 software. The different panels show two representative microscope fields for each of the following conditions: untreated megakaryocytes of patient PD-III **(A)**, megakaryocytes of patient PD-III treated with 0.7% DMSO (negative control) **(B)** and megakaryocytes of patient PD-III treated with the indicated concentrations of G418 **(C)**, ELX-02* **(D)**, PTC-124 **(E)**, 2,6-DAP **(F)** and AMX **(G)**. Scale bar: 10 µm. *The 1000 µM ELX-02 condition is missing due to insufficient availability of ELX-02 when the cells were ready for treatment.



Supplementary Figure S6. Uptake of rescued mutant FV by *ex-vivo* differentiated megakaryocytes of the *F5* p.Arg1161Ter-homozygous patient (low-magnification images). Haematopoietic progenitor cells isolated from peripheral blood of the *F5* p.Arg1161Ter-homozygous patient were cultured in the presence of thrombopoietin and interleukin-3 to induce megakaryocyte differentiation. Starting from day 12, cells were either left unexposed **(A)** or exposed for 4 days to 0.33 nM wild-type FV **(B)**, 0.33 nM mutant FV rescued with G418 **(C)**, or 0.33 nM mutant FV rescued with 2,6-DAP **(D)** in the culture medium, before being processed for immunofluorescence analysis. Cell nuclei were stained with Draq5TM (blue). Internalised FV was visualised using a mouse monoclonal antibody against the light chain of human FV (AHV-5108), followed by a FITC-labelled goat anti-mouse IgG antibody (green). Glass slides were observed under a Leica DMI6000CS fluorescence microscope using a 20x/0.40 dry objective. Images (overlay of blue and green fluorescence channels) were acquired with a DFC365FX camera and analysed using the LAS-AF 3.1.1 software. A representative microscope field is shown for each condition. Scale bar: 20 µm. These images taken at low magnification convey an impression of the percentage of FV-positive cells after exposure to FV in the culture medium.



Supplementary Figure S7. Uptake of rescued mutant FV by *ex-vivo* differentiated megakaryocytes of a FV-deficient patient with missense mutations. Haematopoietic progenitor cells isolated from peripheral blood of FV-deficient patient PD-III, who is compound heterozygous for the *F5* p.Trp255Arg and p.Tyr1623Asp missense mutations[1-3], were cultured in the presence of thrombopoietin and interleukin-3 to induce megakaryocyte differentiation. Starting from day 12, cells were either left unexposed (A) or exposed for 2 days to 0.33 nM wild-type FV (B), 0.33 nM mutant FV rescued with G418 (C), or 0.33 nM mutant FV rescued with 2,6-DAP (D) in the culture medium, before being processed for immunofluorescence analysis. Cell nuclei were stained with Hoechst (blue). Internalised FV was visualised using a mouse monoclonal antibody against the light chain of human FV (AHV-5108), followed by an Alexa FluorTM 647-labelled goat anti-mouse IgG antibody (green). Glass slides were observed under a Leica DMI6000CS fluorescence microscope using a 63x/1.40 oil-immersion objective. Images (overlay of blue and green fluorescence channels) were acquired with a DFC365FX camera and analysed using the LAS-AF 3.1.1 software. Two representative microscope fields are shown for each condition. Scale bar: 10 μm.

REFERENCES

1. Duckers, C., et al., *Low plasma levels of tissue factor pathway inhibitor in patients with congenital factor V deficiency.* Blood, 2008. **112**(9): p. 3615-23.

2. Duckers, C., et al., *Residual platelet factor V ensures thrombin generation in patients with severe congenital factor V deficiency and mild bleeding symptoms.* Blood, 2010. **115**(4): p. 879-86.

3. Radu, C.M., et al., *Endocytosis of exogenous factor V by ex-vivo differentiated megakaryocytes from patients with severe parahaemophilia.* Br J Haematol, 2016. **175**(3): p. 517-524.

CHAPTER 4

Antisense-mediated down-regulation of factor V-short splicing in a liver cell line model

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ABSTRACT

Coagulation factor V (FV) is a liver-derived protein encoded by the *F5* gene. Alternative splicing of *F5* exon 13 produces a low-abundance splicing isoform, known as FV-short, which binds the anticoagulant protein tissue factor pathway inhibitor (TFPIα) with high affinity, stabilising it in the circulation and potently enhancing its anticoagulant activity. Accordingly, rare *F5* gene mutations that up-regulate FV-short splicing are associated with bleeding. In this study we have explored the possibility of decreasing FV-short splicing by antisense-based splicing modulation. To this end, we have designed morpholino antisense oligonucleotides (MAOs) targeting the FV-short-specific donor and acceptor splice sites and tested their efficacy in a liver cell line (HepG2) that naturally expresses full-length FV and FV-short. Cells were treated with 0-20 μM MAO, and full-length FV and FV-short mRNA expression was analysed by RT-(q)PCR. Both MAOs, alone or in combination, decreased the FV-short/full-length FV mRNA ratio down to ~50% of its original value in a specific and dose-dependent manner. This pilot study provides proof-of-principle for the possibility to decrease FV-short expression by antisense-mediated splicing modulation. In turn, this may form the basis for novel therapeutic approaches to bleeding disorders caused by FV-short over-expression and/or elevated TFPIα (activity) levels.

Keywords
Coagulation; Factor V-short; *F5*; alternative splicing; splicing modulation; morpholino; antisense oligonucleotides; bleeding

INTRODUCTION

Eukaryotic protein-coding genes have a unique architecture characterised by alternating coding and non-coding regions, known as exons and introns, respectively. As a gene is transcribed by RNA polymerase II, the primary transcript undergoes splicing, a process in which introns are eliminated and exons are ligated together to form the mature mRNA. These reactions take place in the cell nucleus and are catalysed by a macromolecular ribonucleoprotein complex called spliceosome, which operates according to a splicing code embedded in the pre-mRNA itself. The beginning and end of each intron are marked by relatively conserved sequences, known as donor and acceptor splice sites. Moreover, additional exonic and intronic regulatory elements can promote (enhancers) or inhibit (silencers) splicing at nearby splice sites[1]. Depending on the strength of these signals and on the way they are decoded by the spliceosome, exonic sequence can be excluded from the mature mRNA and/or intronic sequence can be retained, generating different mRNAs from the same primary transcript (alternative splicing). In this way, most human genes can produce multiple isoforms of the same protein[2,3]. Due to the complexity of the splicing code, it is estimated that a substantial fraction of disease-causing genetic variants acts by deregulating splicing[4,5]. Physiological and pathological alternative splicing is amenable to specific modulation by various molecules[6–8]. In particular, antisense oligonucleotides designed to bind by base-pair-complementarity to specific splicing signals on a target pre-mRNA can prevent the recognition of these signals by the spliceosome[9]. Several studies support the ability of this technology to suppress unwanted splicing events (such as aberrant splicing induced by genetic mutations) in favour of alternative (correct or desirable) splicing events (see refs. [10–13] for some coagulation-related examples). In fact, several antisense oligonucleotides have entered clinical trials or have already been approved as therapeutic agents for various diseases[14,15]. Alternative splicing also contributes to the regulation of blood coagulation by generating variants of the same coagulation factor with different functional properties, as in the case of factor V (FV), encoded by the *F5* gene[16]. FV is a liver-derived plasma protein with an A1-A2-B-A3-C1-C2 domain-structure. Its main isoform, referred to as (full-length) FV, is kept inactive by a tight electrostatic interaction between a basic and an acidic region within the large B domain[17]. Step-wise proteolysis of this domain activates FV into the essential cofactor (FVa) of factor Xa (FXa) in the conversion of prothrombin to thrombin, accelerating this reaction over a 1000-fold[18]. In the early stages of coagulation, the procoagulant activity of FV is regulated by the anticoagulant protein tissue factor pathway inhibitor α (TFPIα)[19]. In fact, the C-terminus of TFPIα contains a basic region that can bind to the free acidic region of partially activated forms of FV, thereby preventing their incorporation in the prothrombinase complex[20], as well as their full activation to FVa[21]. In 2013, a minor splicing variant of FV, normally accounting for <5% of plasma FV, was discovered[22]. This so-called FV-short isoform arises from the removal of an optional intron within exon

13 (Figure 1), resulting in the in-frame deletion of 702 amino acids of the B domain, including the basic region. TFPIα binds with high affinity to the unmatched acidic region of FV-short[22], thereby neutralising its constitutive prothrombinase activity[23]. In turn, FV-short stabilises TFPIα in the circulation[22,24] and potently enhances TFPIα-mediated inhibition of FXa in the presence of protein S[25], which binds to both TFPIα and FV-short and strengthens their interaction[26]. FV-short was originally identified in a family with FV-short over-expression due to a *F5* gene mutation that enhances FV-short splicing, resulting in 10-fold increased TFPIα levels and a bleeding tendency (East Texas bleeding disorder)[22,27]. More recently, two other *F5* gene mutations (*F5*-Amsterdam[28] and *F5*-Atlanta[29]) up-regulating FV-short splicing and plasma TFPIα levels have been reported in unrelated patients with similar histories of trauma-related bleeding. As a possible therapeutic approach to these haemorrhagic disorders, we have explored the feasibility of decreasing FV-short expression by antisense-mediated *F5* splicing modulation. To this end, we designed specific antisense oligonucleotides targeting FV-short splicing and tested them *in vitro* in a liver cell line (HepG2) that naturally expresses full-length FV and FV-short.

MATERIALS AND METHODS

Bioinformatics Analysis

The sequence of *F5* exon 13 was analysed in silico for splicing signals using the Human Splicing Finder software[30]. Information on the occurrence of common genetic polymorphisms in the vicinity of the relevant splice sites was retrieved from the Ensembl database (www.ensembl.org, accessed on 10 September 2021).

Antisense Oligonucleotides

Morpholino antisense oligonucleotides (MAOs) are nucleic acid analogues in which DNA bases are bound to a non-charged backbone (morpholine rings linked by phosphorodiamidate bonds)[31]. MAOs targeting the FV-short-specific donor splice site (50-CAAGGTTATTGACAGTGAACTTACT-30, Donor-MAO) and acceptor splice site (5'-AGGTCTGGATAAGGAAAAGACTCAT-3', Acceptor-MAO) were designed. Specificity for the intended target sites was verified by BLAST analysis against the human transcriptome. Another MAO with an irrelevant sequence (5'-CCTCTTACCTCAGTTACAATTTATA-3') was used as a negative control. All MAOs were purchased from GeneTools (Philomath, OR, USA).

Characterisation of the *F5* Gene in HepG2 Cells

Cell Model

HepG2 (ATCC database, accession number HB-8065™) is a well-established human liver

cell line derived from a 15-year-old Caucasian male patient with hepatoblastoma[32,33].

DNA Isolation

Genomic DNA from HepG2 cells was isolated using the Wizard® Genomic DNA Purification Kit (Promega) according to the manufacturer's protocol and quantified with a NanoDrop 2000 (Thermo Scientific Nanodrop Products, Wilmington, DE, USA).

Multiplex Ligation-Dependent Probe Amplification (MLPA)

The *F5* gene copy number in HepG2 cells was determined by MLPA analysis[34] using the SALSA MLPA Probemix P469 *F5* (MRC Holland, Amsterdam, The Netherlands). This kit contains 26 *F5*-specific probes covering all *F5* exons except exon 2, as well as 9 reference probes recognising other autosomic loci. Genomic DNA isolated from HepG2 cells was standardized to 20 ng/µL and the MLPA reaction was performed following the manufacturer's protocol. FAM-labelled MLPA amplification products were mixed with GeneScan™ 600 LIZ® dye Size Standard and Hi-Di™ formamide (Life Technologies, Bleiswijk, The Netherlands), denatured and separated by capillary electrophoresis on an ABI 3730 DNA Analyzer (Life Technologies), as previously described[35]. The results were analysed using Coffalyser.Net (MRC Holland).

Amplification and Sequencing of F5 exon 13

F5 exon 13 was amplified and sequenced using primer sets that collectively cover the whole exon 13 in 7 overlapping amplicons (primers and amplification conditions available on request).

Cell Culture and Treatment

HepG2 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with low glucose (Biowest, Nuaillé, France), supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine. Cells were maintained in an incubator at 37°C and 5% CO_2. Prior to each treatment experiment, the cells were seeded in 6-well plates at ~70% confluency. Cells seeded in 6-well plates were treated with different MAO concentrations (0 - 20 µM) for 48 h in serum-free OptiMEM (Gibco™). MAOs were delivered using Endo-Porter reagent (GeneTools) at a concentration of 6 µM. Three biological replicates of each treatment were performed.

***F5* Transcript Analysis**

RNA isolation and reverse transcription

Total RNA was isolated from HepG2 cells using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions and quantified with a NanoDrop 2000. Total RNA from primary human hepatocytes, which was used as control in some experiments, was purchased from 3H Biomedical AB (Uppsala, Sweden). RNA (2 µg) was reverse-

transcribed with MultiScribe™ Reverse Transcriptase (Applied Biosystems) using a *F5*-specific primer (5'-ATTCCGAGATGCTCTTCATAC-3') located in exon 14.

F5 mRNA Analysis by PCR and Gel Electrophoresis

Due to the extreme differences in size and relative abundance of the full-length FV and FV-short transcripts, the development of a PCR-based protocol for the simultaneous amplification and quantification of both transcripts required extensive optimisation (see Results section). The full-length FV and FV-short transcripts were initially amplified using a common forward primer located in exon 12 (5'-ATGCGTGGAGAATCTGTGAC-3') and transcript-specific reverse primers in exon 13. The reverse primer for the full-length FV transcript (5'-TCTGCAAGGTTATTGACAGTG-3') was located in the portion of exon 13 that is spliced out in FV-short (443-bp amplicon), whereas, the FV-short-specific reverse primer (5'-CATCTGACCAAGGTTAATATTAC-3') was designed on the junction site created by the alternative splicing event (429-bp amplicon). Since the primers for the full-length FV transcript turned out to co-amplify an unexpected alternatively spliced transcript (see Results section), a second set of primers spanning exons 13–14 was designed and used in all subsequent experiments. Forward primers specific for full-length FV (5'-TATCACCTCCTCCAGACCTTG-3', located in the portion of exon 13 that is spliced out in FV-short) and FV-short (5'-TTCTTCCCCAAGTAATATTAACCTTG-3', located across the junction created by the FV-short splicing event), were combined with a common reverse primer in exon 14 (5'-TTCTGGAATATCATCAGAGTCTTCA-3'), yielding PCR products of 473 bp and 397 bp, respectively. To avoid competition for PCR reagents between full-length FV and FV-short mRNA, the two transcripts were amplified in separate PCR reactions run in parallel using the same amplification conditions (denaturation: 30 s at 92°C; annealing: 20 s at 59°C; extension: 25 s at 72°C; repeated for 32 cycles) on a T100 Thermal Cycler (Bio-Rad Laboratories, Veenendaal, The Netherlands). PCR products were analysed by agarose gel electrophoresis (2% agarose) and their identity was confirmed by Sanger sequencing.

Quantification of Alternatively Spliced F5 Transcripts by qPCR

The alternatively spliced *F5* transcripts (full-length FV and FV-short) were quantified by real-time quantitative PCR (qPCR) with SYBR-Green detection using a LightCycler 480 Real-Time PCR instrument (Roche Applied Science). Separate amplification reactions for the full-length FV and FV-short transcripts were carried out using the second set of primers (spanning exons 13–14) described above. Each qPCR reaction was performed in a total volume of 10 µL including 5 µL LightCycler® 480 SYBR Green I Master mix (Roche), 0.5 µM of each primer and 10 ng of cDNA. The qPCR conditions were as follows: Initial denaturation at 95°C for 5 min, 45 cycles of 20 s of denaturation at 95°C, 15 s of annealing at 60°C, and 15 s of extension at 72°C. The specificity of the amplified products was verified by melting curve analysis (65–95°C) and by agarose

gel electrophoresis. qPCR data were analysed using LC480 Conversion (Roche) and LinRegPCR[36] software. Data analysis was carried out by relative quantification using the modified ΔΔCt method, described by Ruijter et al.[36], which takes amplification efficiency into account. All qPCR reactions were carried out in duplicate.

Statistical Analysis

The effects of increasing MAO concentrations on the FV-short/full-length FV mRNA ratio were evaluated with the Jonckheere-Terpstra trend test using the IBM SPSS statistical package. The significance level (α) was set at 0.05.

RESULTS

Bioinformatics Analysis and Antisense Strategy

In silico analysis of the *F5* exon 13 sequence showed that the donor and acceptor splice sites, defining the FV-short specific intron, have relatively high consensus scores of 83% and 88%, respectively (Figure 1). Two MAOs masking these splice sites were designed (Figure 1) and predicted to have no other targets in the human transcriptome. Interrogation of the Ensembl database revealed no common genetic variants in the vicinity of the FV-short splice sites that could potentially weaken the interaction of the MAOs with the target *F5* pre-mRNA.

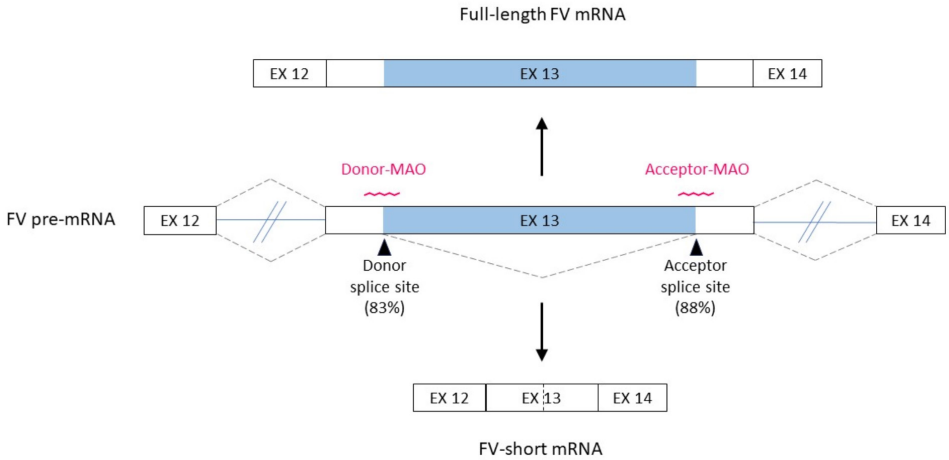
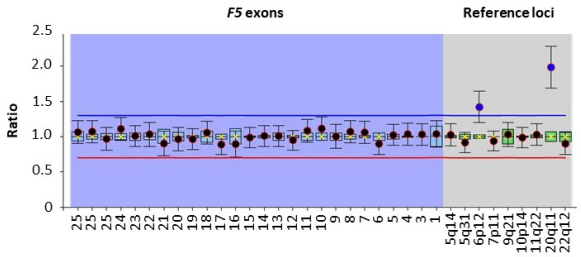


Figure 1. Alternative splicing of the *F5* gene. Schematic representation of the *F5* pre-mRNA region spanning exons 12–14. The large exon 13 contains an internal optional intron (light blue), which is usually retained in the mature mRNA (default full-length FV transcript, top), but occasionally spliced out (alternative FV-short transcript, bottom). The consensus scores for the FV-short-specific donor and acceptor splice sites are reported in brackets and the morpholino antisense oligonucleotides (MAOs) designed to target these sites are shown. EX: exon.

Characterisation of the *F5* gene in HepG2 cells

Since the HepG2 cell line is known to have multiple chromosomal aberrations[33], we performed a preliminary characterisation of the *F5* gene in these cells (Figure 2), in order to ensure the suitability of this model for the intended splicing modulation experiments. The number of copies of the *F5* gene in HepG2 cells was assessed by MLPA analysis and found to be normal (Figure 2A). Two reference probes yielded ratios of 1.5 and 2 in this analysis, indicating the presence of 3 and 4 copies of the corresponding control loci on chromosomes 6 and 20, respectively (Figure 2A). This is consistent with reported copy number changes in the HepG2 genome[33] and unlikely to interfere with the *F5* gene analysis, as the copy numbers of the *F5* gene exons are normalised against the average copy number of all 9 reference probes. Sequencing of the whole *F5* exon 13 from genomic DNA of HepG2 cells identified several well-known single nucleotide polymorphisms (SNPs) (Figure 2B), but no novel sequence variations. Importantly, none of the identified variants was located at or close to the FV-short-specific donor or acceptor splice sites (Figure 2C). Accordingly, preliminary experiments indicated that HepG2 cells express both full-length FV and FV-short mRNA, with a vast predominance of the full-length transcript, as in normal hepatocytes.

A



B

SNP	cDNA position	MAF	HepG2 genotype
rs6016	2208 C/T	0.26	CT
rs6017	2235 T/C	0.26	TC
rs6021	2301 A/G	0.26	AG
rs4524	2573 A/G	0.27	AG
rs4525	2594 A/G	0.26	AG
rs6032	2773 A/G	0.26	GG
rs1800594	3804 T/C	0.31	TC
rs9287090	3948 C/T	0.26	CT
rs9332607	4095 C/T	0.18	CT

C

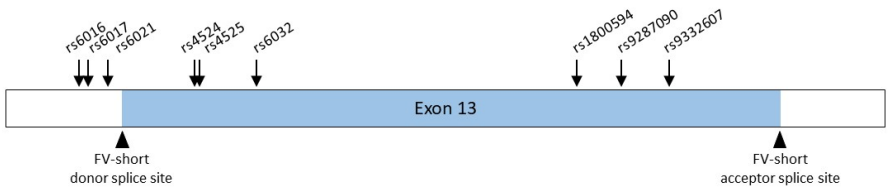


Figure 2. Characterisation of the *F5* gene in HepG2 cells. (A) MLPA analysis of the *F5* gene in HepG2 cells. The normalised copy number is plotted for individual *F5* exons (purple background) and for reference genes chromosomes (grey background). A ratio of ~1 indicates a normal copy number (n = 2), whereas ratios above the blue line or below the red line indicate copy numbers >2 or <2, respectively. **(B)** Common SNPs identified in *F5* exon 13 in the genomic DNA of HepG2 cells; MAF: minor allele frequency. **(C)** Positions of the identified *F5* exon 13 SNPs relative to the FV-short-specific donor and acceptor splice sites.

Optimisation of full-length FV and FV-short transcript amplification and identification of a new alternatively spliced *F5* transcript

In view of determining the effect of MAO treatment on the alternative splicing of *F5*, we developed and optimised an RT-(q)PCR protocol for the simultaneous detection and quantification of the full-length FV and FV-short transcripts, taking into account the extreme differences in the size (~2.1 kb) and relative abundance (>100-fold) of the two transcripts. To this end, we used a *F5*-specific primer (instead of random hexamers) for the reverse-transcription of total RNA, which greatly improved the detection of the low-abundance FV-short transcript. Moreover, to eliminate the bias associated with the higher amplification efficiency of the much shorter FV-short amplicon, we employed transcript-specific primers generating PCR products of similar lengths. Finally, to prevent competition for the PCR reagents between templates of vastly different abundance, we amplified the full-length FV and FV-short transcripts in separate PCR reactions, which were run in parallel using the same thermal cycles. Our original PCR design comprised a common forward primer in exon 12 and transcript-specific primers in exon 13 (red primers in Figure 3A). This set-up worked well for FV-short, but the primer pair for the full-length FV transcript yielded not only the expected product of 443 bp, but also a lesser lower-molecular weight band, which appeared specific, as it could not be eliminated by manipulating the amplification conditions (Figure 3B).

Purification and sequencing of the unexpected PCR product revealed a novel splicing event between the donor splice site of *F5* intron 12 and an alternative acceptor splice site located 123 bp into exon 13 (Figure 3B), leading to the in-frame deletion of the first 123 nucleotides of exon 13. Upon translation, this would predict the loss of 41 amino acids (residues 659–699) between the A2 and B domains of the protein. This alternative splicing event may occur physiologically, as it was also observed in RNA isolated from human primary hepatocytes (data not shown). In silico analysis indicated that the alternative acceptor splice site in exon 13 has a consensus sequence of 83% (vs. 88% for the canonical acceptor splice site at the end of intron 12). To avoid co-amplification of this additional *F5* splicing variant, we switched to a new PCR design based on transcript-specific forward primers in exon 13 and a common reverse primer in exon 14 (black primers in Figure 3A). This revised PCR set-up yielded unique and specific products for both the full-length FV and the FV-short transcript (Figure 3C) and was adopted in all subsequent qualitative and quantitative analyses.

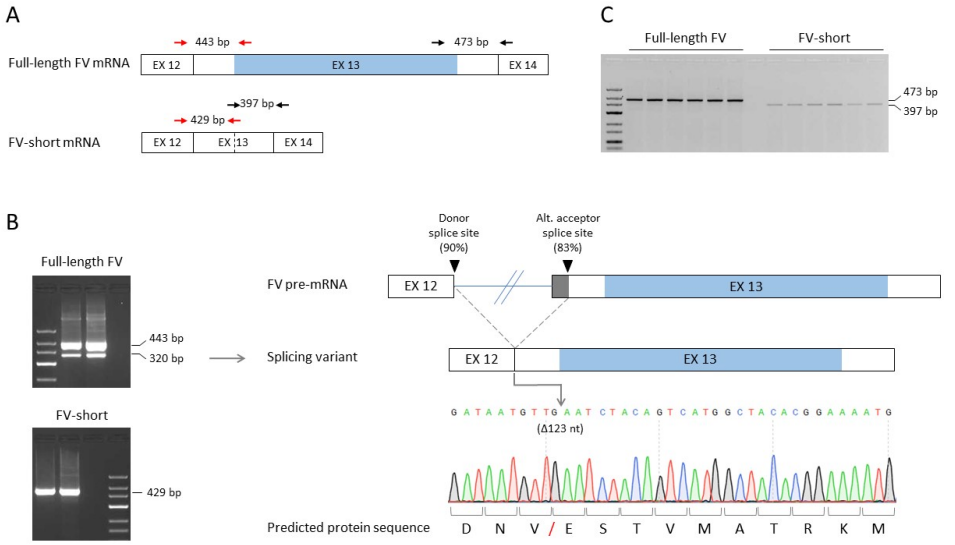


Figure 3. Amplification of FV transcripts and identification of a new alternatively spliced transcript. (A) Schematic representation of the region spanning *F5* exons 12–14 in the full-length FV and FV-short transcripts. The positions of the primers used to amplify these transcripts (first primer set in red, second primer set in black) are shown and amplicon sizes are indicated. EX: exon. **(B)** Left: Typical PCR products obtained after amplification of HepG2 cDNA using the first (red) set of primers (note: since the unexpected 320-bp band was more prominent in qPCR than in normal PCR experiments, the figure shows a gel loaded with qPCR products). Right: Schematic representation and sequencing chromatogram of the new splicing variant lacking the first 123 nucleotides (nt.) of exon 13. The red forward slash in the protein sequence marks the position of the predicted 41-amino acid deletion. **(C)** Typical PCR products obtained after amplification of HepG2 cDNA using the second (black) set of primers.

F5 splicing modulation in HepG2 cells

In order to reduce the relative expression of FV-short, HepG2 cells were treated with increasing concentrations (0–20 M) of MAOs targeting the FV-short-specific donor splice site (Donor-MAO) or acceptor splice site (Acceptor-MAO). After 48 h of treatment, total RNA was isolated and full-length FV and FV-short mRNA expression was analysed by RT-PCR and gel electrophoresis (Figure 4). Both MAOs caused a dose-dependent decrease of FV-short expression without affecting full-length FV expression. Similar results were obtained with a 1:1 mix of Donor-MAO and Acceptor-MAO (MAO-mix, i.e., 50% Donor-MAO and 50% of Acceptor-MAO to obtain an overall concentration of 5 μM, 10 μM and 20 μM), whereas treatment with a Control-MAO up to a concentration of 20 μM did not affect FV-short or full-length FV expression. The effects of the MAOs on the FV-short/full-length FV transcript ratio were quantified using real-time qPCR analysis (Figure 5). Untreated HepG2 cells typically expressed 2–3 orders of magnitude more full-length FV than FV-short mRNA. Treatment with the Donor-MAO dose-dependently decreased the FV-short/full-length FV transcript ratio to ~50% of its original value in

untreated cells (Figure 5A, p-for-trend = 0.008). The Acceptor-MAO was somewhat less effective, reducing the FV-short/full-length FV transcript ratio by ~40% at the maximal dose of 20 μM (Figure 5B, p-for-trend = 0.063). The MAO-mix showed an intermediate effect (Figure 5C, p-for-trend = 0.010), while the Control-MAO did not decrease the FV-short/full-length FV transcript ratio (Figure 5D, p-for-trend = n.s.). Increasing the concentration of Donor-MAO or Acceptor-MAO to 50 μM did not result in any further decrease of the FV-short/full-length FV ratio (not shown).

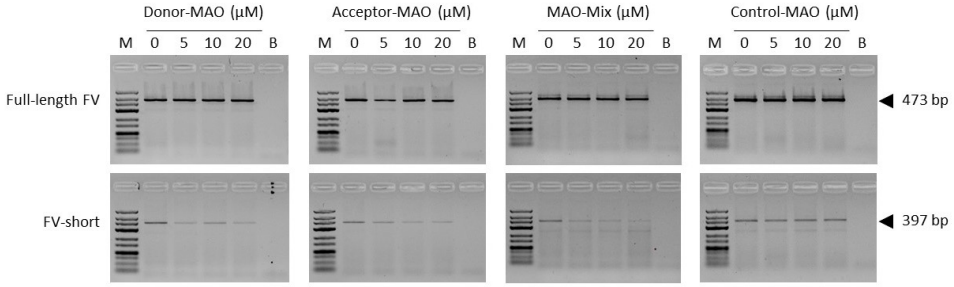


Figure 4. Qualitative analysis of the full-length FV and FV-short transcripts in untreated and treated HepG2 cells. HepG2 cells were treated for 48 h with increasing concentrations (0–20 μM) of a morpholino antisense oligonucleotide (MAO) targeting the FV-short-specific donor splice site (Donor-MAO), a MAO targeting the FV-short-specific acceptor splice site (Acceptor-MAO), a 1:1 mix of Donor-MAO and Acceptor-MAO, (MAO-mix), or a control MAO with an irrelevant sequence. Total RNA was isolated and reverse transcribed into cDNA. Amplicons corresponding to the full-length FV (top) and FV-short transcripts (bottom) were amplified in separate PCR reactions and PCR products were analysed by gel electrophoresis. M: molecular weight marker, B: blank. Sequencing chromatograms of the full-length FV and FV-short amplicons are shown in Supplementary Figure S1.

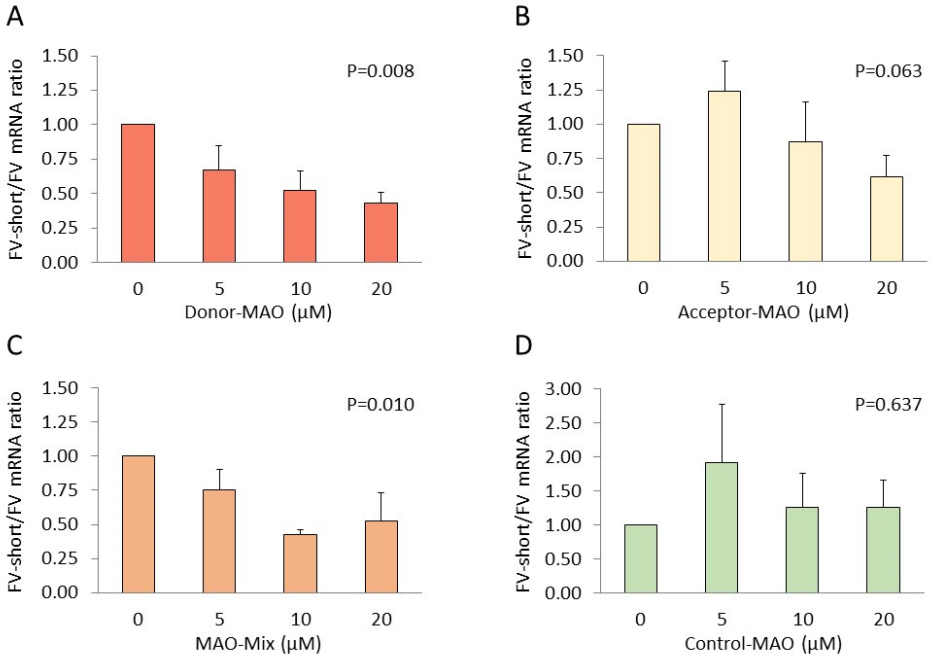


Figure 5. Quantitative analysis of full-length FV and FV-short transcripts in untreated and treated HepG2 cells. HepG2 cells were treated with 0–20 µM morpholino antisense oligonucleotides (MAOs) for 48 h. Total RNA was isolated and reverse-transcribed into cDNA, and the full-length FV and FV-short transcripts were quantified by real-time qPCR analysis. The FV-short/full-length FV mRNA ratio, normalised to the FV-short/full-length FV ratio of untreated cells, was plotted as a function of the concentration of **(A)** a MAO targeting the FV-short-specific donor splice site (Donor-MAO), **(B)** a MAO targeting the FV-short-specific acceptor splice site (Acceptor-MAO), **(C)** a 1:1 mix of Donor-MAO and Acceptor-MAO (MAO-mix), and **(D)** a control MAO with an irrelevant sequence. Results are reported as mean ± S.E.M. of three biological replicates (two for the Control-MAO). The numbers in the top right corner of each graph represent the p-for-trend (Jonckheere-Terpstra trend test) of the respective titrations. Note the different y-axis scale for Control-MAO.

DISCUSSION

F5 gene mutations that up-regulate FV-short splicing are associated with a bleeding tendency, such as in the recently characterised East Texas[22], Amsterdam[28] and Atlanta[29] bleeding disorders. This is due to the major increase in the level and anticoagulant activity of plasma TFPIα that accompanies FV-short over-expression[22]. Currently, there is no specific treatment for these bleeding disorders and their clinical management remains challenging. Affected patients have been treated with plasma and/or prothrombin complex concentrates[22,28], but therapies specifically targeting TFPIα (such as those

used as bypassing agents for haemophilia) might be more appropriate. Although several aptamers, antibodies and peptides against TFPIα are already in various stages of development as therapeutic agents[37], in this study we propose an alternative strategy based on the use of antisense oligonucleotides to down-regulate FV-short splicing. The rationale for this approach is that elevated FV-short is the primary abnormality in the East Texas and allied bleeding disorders, and that FV-short is both the carrier of TFPIα in plasma[22] and a potent cofactor for its activity[25]. Therefore, down-regulating FV-short might be even more effective than antagonising TFPIα directly. Moreover, the liver, which is the main site of FV biosynthesis, is considered an optimal target for splicing modulation therapies[38], and MAOs generally show high bioavailability and low toxicity profiles in *in vivo* studies[39].

In order to decrease FV-short splicing, we have targeted the donor and acceptor splice sites that define the FV-short-specific intron. This was a somewhat necessary choice, because enhancers and silencers potentially regulating FV-short splicing are presently unknown and, due to their rather degenerate consensus sequences, cannot be easily predicted in silico. However, splicing regulatory elements are likely to play an important role in the physiological control of FV-short splicing, as suggested by (a) the very low *in vivo* expression of FV-short despite the relatively high consensus scores (>80%) of the FV-short-specific donor and acceptor splice sites; and (b) the massive up-regulation of FV-short splicing associated with the *F5*-Atlanta deletion[29], which presumably acts by eliminating one or more splicing silencers or by bringing a splicing enhancer closer to the FV-short donor splice site[40]. Therefore, future elucidation of these additional splicing signals may suggest novel and better targets for antisense-mediated FV-short splicing modulation.

The ability of our MAOs to decrease FV-short splicing was tested *in vitro* in HepG2 cells, which express both full-length FV and FV-short mRNA in proportions similar to normal hepatocytes. Both MAOs, alone or in combination, dose-dependently decreased the FV-short/full-length FV ratio down to ~50% of its original value in the absence of treatment. Some scattering in the qPCR data might be attributed to the very low concentration of the FV-short mRNA already before treatment, and the consequent extremely narrow dynamic range following treatment. The effects of the two MAOs were specific, but (contrary to our expectation) not synergistic. To produce an appreciable change in the overall splicing pattern, MAOs need to access a substantial fraction of cells and to end up in the nucleus. Although MAO transfection efficiency was unfortunately not evaluated in our study, previous work from our laboratory suggests that the Endo-Porter reagent is able to effectively deliver MAOs to HepG2 cells, as judged by the high efficiency of splicing correction achieved upon treatment with a mutation-specific MAO[11]. Moreover, the analysis of the *F5* gene in

HepG2 cells excluded *F5* gene amplification, as well as the presence of sequence variations that could interfere with the annealing of the MAOs to their intended target sites on the *F5* pre-mRNA. Therefore, we speculate that the inability of our MAOs to reduce FV-short expression below 50% (even at a concentration of 50 μM) may be related to the complexity of alternative splicing regulation[41], which involves many other mechanisms (epigenetics, transcription rate, pre-mRNA secondary structures, etc.) and regulatory elements (splicing enhancers and silencers, trans-acting splicing factors) than just the donor and acceptor splice sites. Since the FV-short concentration in conditioned media was too low for detection by Western blot analysis and no other assay is currently available to measure FV-short, we could unfortunately not verify whether the observed reduction in FV-short mRNA upon MAO treatment also translates in a decrease in FV-short protein expression. This is a major limitation of our study. Moreover, it should be emphasised that, unlike the East Texas[22], Amsterdam[28] and Atlanta[29] patients, the HepG2 cell model employed in our study has a normal *F5* exon 13 sequence and a very low basal expression of FV-short. Therefore, it remains to be established whether an antisense-mediated approach using mutation-specific MAOs would be able to correct FV-short over-expression in models of these diseases, such as patients' induced pluripotent stem cell (iPSC)-derived hepatocytes. On the other hand, it is worth noting that FV-short levels show major inter-individual differences even in the absence of *F5* gene mutations, and that high levels and/or activity of TFPIα contribute to the haemorrhagic tendency in various coagulopathies, from haemophilia[42] to bleeding arising from unknown causes[43,44]. Therefore, antisense-mediated strategies aimed at decreasing FV-short splicing might be more widely applicable than just to the East Texas and allied disorders. As a by-product of this study, the carefully optimised (q)PCR-based protocol for the detection and quantification of the FV-short transcript may find application in the analysis of this splicing variant in total RNA samples from primary hepatocytes and other cell types under various (pathological) conditions. Since the *F5* gene is robustly transcribed in blood cells, FV-short transcript levels determined in whole blood RNA might serve as a surrogate marker for protein levels until a FV-short ELISA becomes available. Finally, we have serendipitously identified a new *F5* splicing event between exons 12 and 13, which eliminates the first 123 nucleotides of exon 13, predicting the in-frame deletion of amino acids 659–699 between the A2 and B domains of FV. Whether the low-abundance alternatively spliced transcript containing this deletion is also translated into protein is currently unknown. Interestingly, this alternative splicing event is yet different from the one recently reported as an incidental finding in the *F5*-Atlanta paper[29], further underscoring the variety and complexity of the splicing pattern of this region of the *F5* gene.

CONCLUSIONS

In conclusion, this pilot study provides *in vitro* proof-of-principle for the possibility to specifically down-regulate FV-short mRNA expression using MAOs targeting the donor and acceptor splice sites of the FV-short-specific intron. Additional work is needed to validate these findings at the protein level and to extend them to more relevant models of FV-short over-expression. Considering the dual function of FV-short as a carrier and cofactor of TFPIα, antisense-based down-regulation of FV-short splicing may eventually create opportunities for novel therapeutic avenues for various bleeding disorders caused by elevated TFPIα (activity) levels.

REFERENCES

1. Wang, Z. and C.B. Burge, *Splicing regulation: from a parts list of regulatory elements to an integrated splicing code.* RNA, 2008. **14**(5): p. 802-13.
2. Kornblihtt, A.R., et al., *Alternative splicing: a pivotal step between eukaryotic transcription and translation.* Nat Rev Mol Cell Biol, 2013. **14**(3): p. 153-65.
3. Nilsen, T.W. and B.R. Graveley, *Expansion of the eukaryotic proteome by alternative splicing.* Nature, 2010. **463**(7280): p. 457-63.
4. Li, Y.I., et al., *RNA splicing is a primary link between genetic variation and disease.* Science, 2016. **352**(6285): p. 600-4.
5. Lord, J. and D. Baralle, *Splicing in the Diagnosis of Rare Disease: Advances and Challenges.* Front Genet, 2021. **12**: p. 689892.
6. Hammond, S.M. and M.J. Wood, *Genetic therapies for RNA mis-splicing diseases.* Trends Genet, 2011. **27**(5): p. 196-205.
7. Black, A.J., J.R. Gamarra, and J. Giudice, *More than a messenger: Alternative splicing as a therapeutic target.* Biochim Biophys Acta Gene Regul Mech, 2019. **1862**(11-12): p. 194395.
8. Balestra, D. and A. Branchini, *Molecular Mechanisms and Determinants of Innovative Correction Approaches in Coagulation Factor Deficiencies.* Int J Mol Sci, 2019. **20**(12).
9. Havens, M.A. and M.L. Hastings, *Splice-switching antisense oligonucleotides as therapeutic drugs.* Nucleic Acids Res, 2016. **44**(14): p. 6549-63.
10. Davis, R.L., et al., *A deep intronic mutation in FGB creates a consensus exonic splicing enhancer motif that results in afibrinogenemia caused by aberrant mRNA splicing, which can be corrected in vitro with antisense oligonucleotide treatment.* Hum Mutat, 2009. **30**(2): p. 221-7.
11. Nuzzo, F., et al., *Antisense-based RNA therapy of factor V deficiency: in vitro and ex vivo rescue of a F5 deep-intronic splicing mutation.* Blood, 2013. **122**(23): p. 3825-31.
12. Nuzzo, F., et al., *Characterization of an apparently synonymous F5 mutation causing aberrant splicing and factor V deficiency.* Haemophilia, 2015. **21**(2): p. 241-8.
13. Balestra, D., et al., *Regulation of a strong F9 cryptic 5'ss by intrinsic elements and by combination of tailored U1snRNAs with antisense oligonucleotides.* Hum Mol Genet, 2015. **24**(17): p. 4809-16.
14. Levin, A.A., *Treating Disease at the RNA Level with Oligonucleotides.* N Engl J Med, 2019. **380**(1): p. 57-70.
15. Crooke, S.T., et al., *Antisense technology: A review.* J Biol Chem, 2021. **296**: p. 100416.
16. Dahlbäck, B., *Pro- and anticoagulant properties of factor V in pathogenesis of thrombosis and bleeding disorders.* Int J Lab Hematol, 2016. **38**(Suppl. 1): p. 4-11.
17. Bos, M.H.A. and R.M. Camire, *A Bipartite Autoinhibitory Region within the B-domain Suppresses Function in Factor V.* J Biol Chem, 2012. **287**(31): p. 26342-26351.
18. Rosing, J., et al., *The role of phospholipids and factor Va in the prothrombinase complex.* J Biol Chem, 1980. **255**(1): p. 274-83.
19. Wood, J.P., et al., *Biology of tissue factor pathway inhibitor.* Blood, 2014. **123**(19): p. 2934-2943.
20. Wood, J.P., et al., *Tissue factor pathway inhibitor-alpha inhibits prothrombinase during the initiation of blood coagulation.* Proc Natl Acad Sci U S A, 2013. **110**(44): p. 17838-43.
21. van Doorn, P., et al., *The C-terminus of tissue factor pathway inhibitor-alpha inhibits factor V activation by protecting the Arg1545 cleavage site.* J Thromb Haemost, 2017. **15**(1): p. 140-149.
22. Vincent, L.M., et al., *Coagulation factor V(A2440G) causes east Texas bleeding disorder via TFPIα.* J Clin Invest, 2013. **123**(9): p. 3777-3787.
23. Petrillo, T., et al., *Regulation of factor V and factor V-short by TFPIalpha: Relationship between B-domain proteolysis and binding.* J Biol Chem, 2021. **296**: p. 100234.

24. Duckers, C., et al., *Low plasma levels of tissue factor pathway inhibitor in patients with congenital factor V deficiency.* Blood, 2008. **112**(9): p. 3615-23.

25. Dahlbäck, B., et al., *Factor V-short and protein S as synergistic tissue factor pathway inhibitor (TFPIalpha) cofactors.* Res Pract Thromb Haemost, 2018. **2**(1): p. 114-124.

26. Dahlbäck, B. and S. Tran, *The preAR2 region (1458-1492) in Factor V-Short is crucial for the synergistic TFPIalpha-cofactor activity with protein S and the assembly of a trimolecular Factor Xa-inhibitory complex comprising FV-Short, protein S and TFPIalpha.* J Thromb Haemost, 2021.

27. Kuang, S.Q., et al., *Characterization of a novel autosomal dominant bleeding disorder in a large kindred from east Texas.* Blood, 2001. **97**(6): p. 1549-54.

28. Cunha, M.L., et al., *A novel mutation in the F5 gene (factor V Amsterdam) associated with bleeding independent of factor V procoagulant function.* Blood, 2015. **125**(11): p. 1822-5.

29. Zimowski, K.L., et al., *F5-Atlanta: A novel mutation in F5 associated with enhanced East Texas splicing and FV-short production.* J Thromb Haemost, 2021. **19**(7): p. 1653-1665.

30. Desmet, F.O., et al., *Human Splicing Finder: an online bioinformatics tool to predict splicing signals.* Nucleic Acids Res, 2009. **37**(9): p. e67.

31. Summerton, J. and D. Weller, *Morpholino antisense oligomers: design, preparation, and properties.* Antisense Nucleic Acid Drug Dev, 1997. **7**(3): p. 187-95.

32. Lopez-Terrada, D., et al., *Hep G2 is a hepatoblastoma-derived cell line.* Hum Pathol, 2009. **40**(10): p. 1512-5.

33. Zhou, B., et al., *Haplotype-resolved and integrated genome analysis of the cancer cell line HepG2.* Nucleic Acids Res, 2019. **47**(8): p. 3846-3861.

34. Schouten, J.P., et al., *Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification.* Nucleic Acids Res, 2002. **30**(12): p. e57.

35. Nuzzo, F., et al., *Identification of a novel large deletion in a patient with severe factor V deficiency using an in-house F5 MLPA assay.* Haemophilia, 2015. **21**(1): p. 140-7.

36. Ruijter, J.M., et al., *Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data.* Nucleic Acids Res, 2009. **37**(6): p. e45.

37. Chowdary, P., *Anti-tissue factor pathway inhibitor (TFPI) therapy: a novel approach to the treatment of haemophilia.* Int J Hematol, 2020. **111**(1): p. 42-50.

38. Sehgal, A., A. Vaishnaw, and K. Fitzgerald, *Liver as a target for oligonucleotide therapeutics.* J Hepatol, 2013. **59**(6): p. 1354-9.

39. Amantana, A. and P.L. Iversen, *Pharmacokinetics and biodistribution of phosphorodiamidate morpholino antisense oligomers.* Curr Opin Pharmacol, 2005. **5**(5): p. 550-5.

40. Castoldi, E., *F5-Atlanta: Factor V-short strikes again.* J Thromb Haemost, 2021. **19**(7): p. 1638-1640.

41. Shenasa, H. and K.J. Hertel, *Combinatorial regulation of alternative splicing.* Biochim Biophys Acta Gene Regul Mech, 2019. **1862**(11-12): p. 194392.

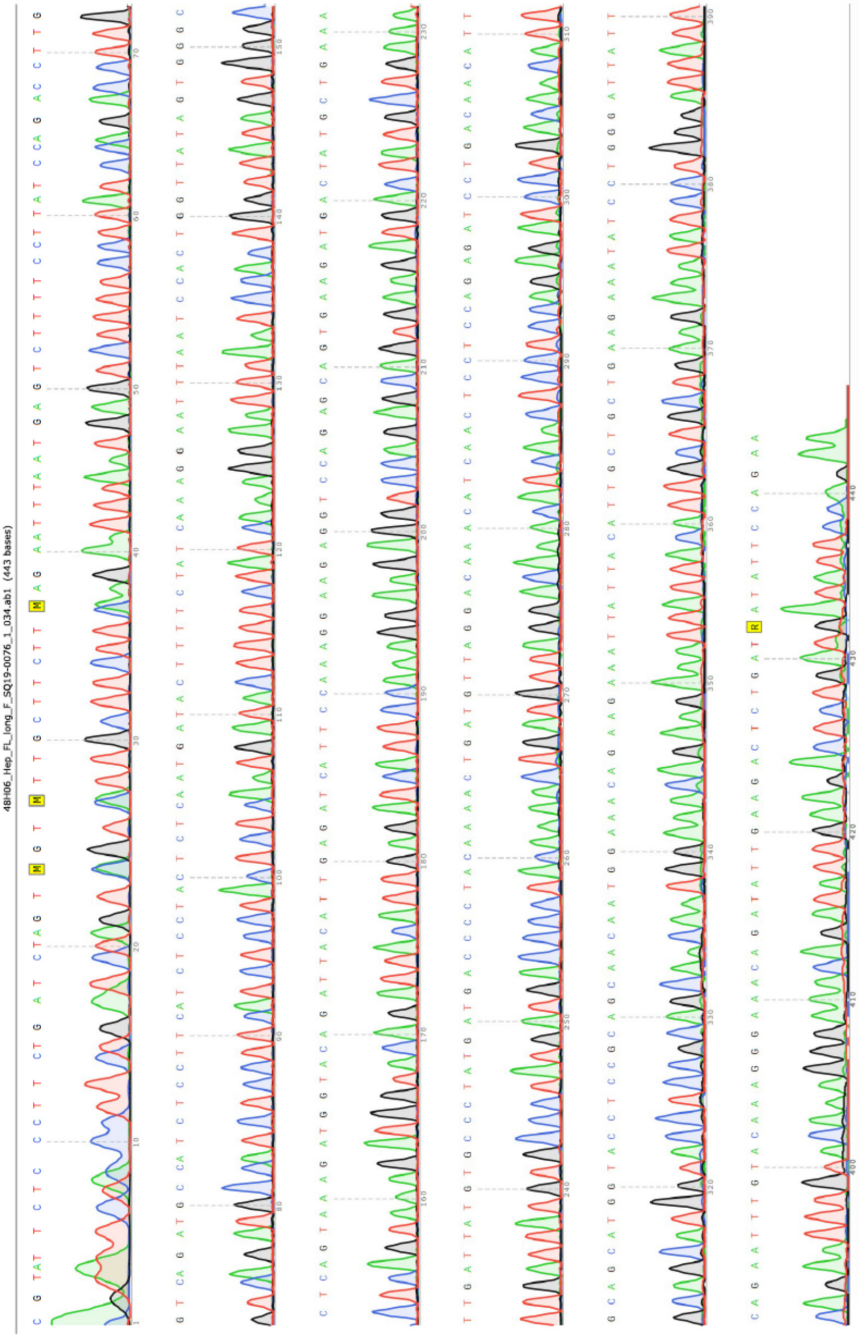
42. Chelle, P., et al., *Tissue factor pathway inhibitor is the main determinant of thrombin generation in haemophilic patients.* Haemophilia, 2019. **25**(2): p. 343-348.

43. MacDonald, S., et al., *Investigation of patients with unclassified bleeding disorder and abnormal thrombin generation for physiological coagulation inhibitors reveals multiple abnormalities and a subset of patients with increased tissue factor pathway inhibitor activity.* Int J Lab Hematol, 2020. **42**(3): p. 246-255.

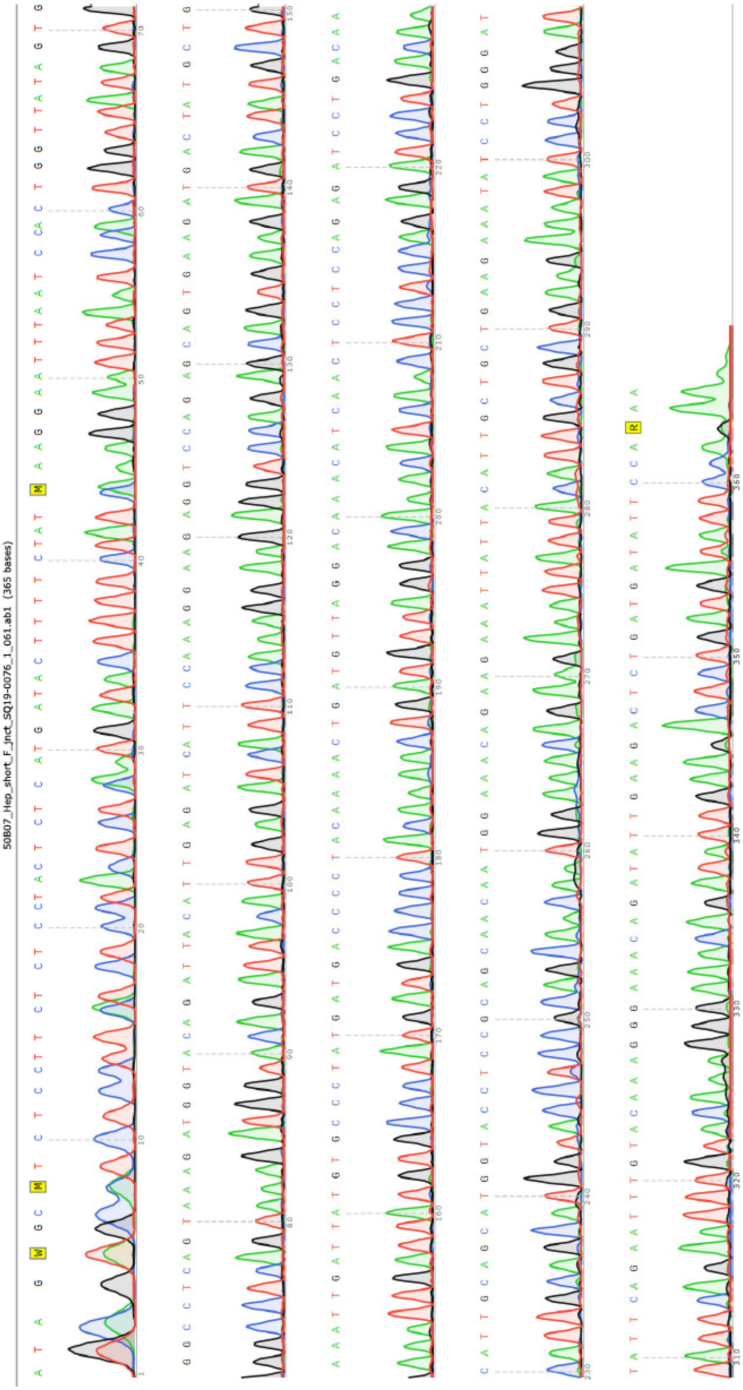
44. Mehic, D., et al., *Elevated levels of tissue factor pathway inhibitor in patients with mild to moderate bleeding tendency.* Blood Adv, 2021. **5**(2): p. 391-398.

SUPPLEMENTARY MATERIALS

(A) Sequencing chromatogram of the 473-bp band corresponding to the full-length FV transcript shown in Figure 4 of the manuscript.



(B) Sequencing chromatogram of the 397-bp band corresponding to the FV-short transcript shown in Figure 4 of the manuscript.



CHAPTER 5

Antisense approaches to down-regulate FV-short expression in FV-short-related bleeding disorders

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Preliminary report

CHAPTER 6

General discussion

CHAPTER 7

Summary
Nederlandse samenvatting

SUMMARY

Haemostasis is a finely regulated process that prevents excessive bleeding or clotting. However, genetic mutations in haemostasis-related genes can disrupt this delicate equilibrium, resulting in a bleeding disorder or a thrombotic event.

This thesis focusses on specific genetic defects responsible for bleeding disorders as potential targets for molecular therapeutic approaches. In particular, this thesis focusses on FV and FV-short and on VWF.

Chapter 1 provides an introduction on the mechanisms of haemostasis to stop the bleeding and to prevent blood loss in case of vascular damage. In addition, it describes the bleeding disorders caused by defects of VWF and FV. This chapter also introduces FV-short, a splicing isoform of FV, and the bleeding disorders associated with FV-short over-expression.

Chapter 2 describes a patient with von Willebrand disease (VWD) carrying two *VWF* variants: a novel mutation (R760S) and a common SNP (R924Q). The R760S mutation was extensively characterised and investigated *in vitro*. *In silico* model suggested that the R760S mutation could interfere with cleavage of the propeptide from pre-pro-VWF and this was demonstrated experimentally. In addition, persistence of the propeptide was found to interfere with VWF-FVIII binding. *VWF* mRNA analysis in the patient confirmed the presence of the two mutations in *cis* and revealed that only the mutated allele was expressed. The 'wild type' allele appeared not to be expressed. The underlying cause is still under investigation.

Chapter 3 investigates a patient with FV-deficiency due to a nonsense mutation in *F5* exon 13. The proband is homozygous for this nonsense mutation and has a moderate bleeding tendency. This type of mutation may respond to treatment with agents that enhance the natural readthrough. Analysis of FV (mRNA and protein) resulted favourable for a readthrough correction approach, thus *in vitro* and *ex vivo* experiments were carried out to rescue the impaired FV. COS-1 cells were transfected with *F5* cDNA carrying the nonsense mutation and treated with 5 different agents that were shown in literature to enhance readthrough. *In vitro*, three agents proved able to rescue the expression of functional FV. A cytotoxicity assay performed on three different cell lines revealed that all compounds have some degree of cytotoxicity, except ELX-02. In the *ex vivo* model, patient's megakaryocytes were differentiated from peripheral blood stem cells and treated with the same five compounds. Upon treatment, FV expression in the patient's megakaryocytes was restored. In addition, megakaryocytes were able to internalise G418-corrected mutant FV and DAP-corrected mutant FV, a key finding

for the clinical application of these agents.

Chapter 4 introduces FV-short, the isoform of FV arising from alternative splicing of exon 13 of the *F5* mRNA, resulting in an in-frame deletion of 702 amino acids of the B domain. FV-short tightly binds TFPIα, stabilising it in the circulation and enhancing its inhibitory activity. Mutations that enhance FV-short expression (FV-East Texas, FV-Amsterdam and FV-Atlanta) cause higher levels of TFPIα, resulting in bleeding disorders. In this chapter we explore the possibility of downregulating FV-short using morpholino antisense oligonucleotides (MAOs). A liver cell line (HepG2) that naturally expresses FV and FV-short was used as model. Genetic analysis of HepG2 cells showed a normal *F5* gene copy number; common SNPs were identified but none of them was located at or close to FV-short donor and acceptor splice sites. Furthermore, HepG2 cells express more FV than FV-short, just like normal hepatocytes. Specific MAOs targeting donor or acceptor splice site of FV-short were designed and tested for their ability to downregulate FV-short. Upon 48 h treatment with increasing doses of MAOs (0-50 μM), both MAOs, alone or in combination, decreased the expression of FV-short mRNA. Since an assay to measure FV-short is not yet available, a qPCR was designed and carefully optimised to quantify FV-short mRNA.

In **chapter 5** we report preliminary results on the use of specific MAOs and siRNAs to downregulate FV-short in *in vitro* models of FV-short over-expression. COS-1 cells were transiently transfected with WT *F5* cDNA or with *F5* cDNAs carrying the FV-East Texas, FV-Amsterdam or FV-Atlanta mutations. FV-short expression in transfected cells was comparable to the FV-short expression found in patients carrying the corresponding mutation. Specific MAOs targeting the donor and acceptor splice sites of each variant were designed and tested, but they did not result in FV-short mRNA downregulation. Differently, siRNAs designed to specifically target the unique FV-short junction created by the alternative splicing event resulted in dose-dependent downregulation of FV-short mRNA expression.

Chapter 6 integrates all the findings of this thesis and discusses them in the light of current literature. Conclusions and future perspectives are given.

NEDERLANDSE SAMENVATTING

Hemostase is een nauwkeurig gereguleerd proces dat overmatig bloeden of stollen voorkomt. Genetische mutaties in hemostasegerelateerde genen kunnen dit delicate evenwicht echter verstoren, wat kan leiden tot een bloedingsstoornis of een trombose. Dit proefschrift richt zich op het bestuderen van specifieke genetische mutaties die verantwoordelijk zijn voor bloedingsstoornissen als potentiële target voor moleculair therapeutische benaderingen. In het bijzonder richt dit proefschrift zich op FV en FV-short en op VWF.

In **hoofdstuk 1** wordt een inleiding gegeven over de mechanismen van hemostase om bloedverlies te voorkomen in geval van vasculaire schade. Daarnaast beschrijft het de bloedingsstoornissen veroorzaakt door defecten in VWF en FV. In dit hoofdstuk wordt FV-short geintroduceerd, een splicing-isovorm van FV, en de bloedingsstoornissen die geassocieerd zijn met overexpressie van FV-short.

In **hoofdstuk 2** wordt een patiënte met de ziekte van von Willebrand (VWD) beschreven die drager is van twee VWF genetische varianten: een nieuwe mutatie (R760S) en een veel voorkomend polymorfisme (R924Q). De R760S-mutatie werd *in vitro* uitgebreid gekarakteriseerd en onderzocht. Het *in silico*-model suggereerde dat de R760S-mutatie de splitsing van het propeptide van pre-pro-VWF zou kunnen verstoren en dit werd experimenteel aangetoond. Bovendien bleek de persistentie van het propeptide de binding van VWF-FVIII te verstoren. VWF-mRNA-analyse bij de patiënte bevestigde de aanwezigheid van de twee mutaties in cis en onthulde dat alleen het gemuteerde allel tot expressie kwam. Het 'wild type' allel bleek niet tot expressie te komen. De onderliggende oorzaak wordt nog onderzocht.

In **hoofdstuk 3** wordt een patiënt beschreven met FV-deficiëntie als gevolg van een nonsense mutatie in F5 exon 13. De proband is homozygoot voor deze nonsense mutatie en heeft een matige bloedingsneiging. Dit type mutatie kan reageren op behandeling met middelen die de natuurlijke readthrough versterken. Analyse van FV (mRNA en eiwit) resulteerde gunstig voor een readthrough-correctiebenadering, dus werden *in vitro* en *ex vivo* experimenten uitgevoerd om de expressie van FV te herstellen. COS-1-cellen werden getransfecteerd met *F5*-cDNA dat de nonsense-mutatie draagt en behandeld met 5 verschillende middelen waarvan in de literatuur werd aangetoond dat ze de readthrough versterken. *In vitro* bleken drie middelen in staat de expressie van functionele FV enigszins te herstellen. Een cytotoxiciteitstest uitgevoerd op drie verschillende cellijnen onthulde dat alle geteste middelen een zekere mate van cytotoxiciteit hebben, behalve ELX-02. In het *ex vivo* model werden stamcellen verkregen uit perifeer bloed van de patiënt naar megakaryocyten gedifferentieerd en vervolgens

behandeld met dezelfde vijf middelen. Na de behandeling werd de FV expressie in de megakaryocyten van de patiënt hersteld. Bovendien waren megakaryocyten in staat om G418-gecorrigeerde mutante FV en DAP-gecorrigeerde mutante FV te internaliseren, een belangrijke bevinding voor de klinische toepassing van deze middelen.

In **hoofdstuk 4** wordt FV-short geïntroduceerd, de isovorm van FV die voortkomt uit alternatieve splicing van exon 13 van het *F5*-mRNA, resulterend in een in-frame deletie van 702 aminozuren van het B-domein. FV-short bindt stevig aan TFPIα, stabiliseert het in de circulatie en versterkt de antistollende activiteit ervan. Mutaties die de FV-short-expressie verhogen (FV-East Texas, FV-Amsterdam en FV-Atlanta) veroorzaken hogere niveaus van TFPIα, resulterend in bloedingsstoornissen. In dit hoofdstuk onderzoeken we de mogelijkheid om de expressie van FV-short te verminderen met behulp van morfolino antisense oligonucleotiden (MAO's). Als model werd een levercellijn (HepG2) gebruikt die van nature FV en FV-short tot expressie brengt. Genetische analyse van HepG2-cellen toonde een normaal aantal kopieen van het *F5*-gen aan; veelvoorkomende polymorfismen werden geïdentificeerd, maar geen van hen bevond zich op of dichtbij de FV-short-specifieke donor- en acceptor- splicing sites. Bovendien brengen HepG2-cellen meer FV tot expressie dan FV-short, net als primaire hepatocyten. Specifieke MAO's die de donor- of acceptor- splicing sites van FV-short kunnen afdekken werden ontworpen en getest op hun vermogen om FV-short splicing te downreguleren. Na 48 uur behandeling met toenemende doses MAO's (0-50 μM) zorgden beide MAO's, alleen of in combinatie, voor verminderde expressie van FV-short mRNA. Omdat er nog geen test beschikbaar is om FV-short eiwit te meten, werd een qPCR ontworpen en zorgvuldig geoptimaliseerd om FV-short-mRNA te kwantificeren.

In **hoofdstuk 5** rapporteren we voorlopige resultaten over het gebruik van specifieke MAO's en siRNA's om FV-short expressie te verminderen in *in vitro* modellen van overexpressie van FV-short. COS-1-cellen werden tijdelijk getransfecteerd met WT *F5*-cDNA of met *F5*-cDNA's die de FV-East Texas-, FV-Amsterdam- of FV-Atlanta-mutaties dragen. FV-short expressie in getransfecteerde cellen was vergelijkbaar met de FV-short expressie gevonden bij patiënten die de overeenkomstige mutatie dragen. Specifieke MAO's gericht op de donor- en acceptor- splicing sites van elke variant werden ontworpen en getest, maar ze resulteerden niet in vermindering van FV-short mRNA. Echter, siRNA's die zijn ontworpen om zich specifiek te richten op de unieke FV-short sequentie gecreëerd door de alternatieve splicing, resulteerden in dosisafhankelijke vermindering van FV-short mRNA-expressie.

In **hoofdstuk 6** worden alle bevindingen van dit proefschrift geïntegreerd en bespreekt deze in het licht van de huidige literatuur. Er worden conclusies en toekomstperspectieven gegeven.

CHAPTER 8

Impact

The human body physiologically possesses an innate ability to prevent excessive bleeding and thrombotic episodes. These two mechanisms are constantly balancing each other in haemostasis. Mutations in genes involved in haemostasis may perturb this delicate equilibrium, thereby predisposing to bleeding or thrombosis.

This thesis focusses on genetic variants responsible for inherited bleeding disorders with the aim of designing personalised therapies to specifically target the cause of the disorder.

For FV deficiency there is currently no replacement therapy available. The treatment and prophylaxis of these patients relies on plasma transfusion. This treatment requires hospitalisation and, despite the extraordinary advances in transfusion medicine, might expose patients to risks such as allergic reactions and overload of the cardiovascular system. Therefore, we investigated in a pre-clinical setting known and new molecules as possible therapeutics for FV deficiency caused by a specific nonsense mutation. We demonstrated that this nonsense mutation has favourable characteristics for correction. This approach has the potential to be extended to other nonsense mutations with the same favourable characteristics. One of these molecules (PTC-124), which has an oral route of administration, has been approved for the treatment of Duchenne muscular dystrophy due to nonsense mutations. A potential approval for FV-deficient patients would alleviate the burden of repeated transfusion and ameliorate their quality of life. In this thesis we also investigated molecular approaches to modify the expression of FV-short, a low-abundance isoform of FV. The overexpression of FV-short is responsible for increased levels of an important anticoagulant protein (TFPI), causing the bleeding diathesis. To this date, 4 families with genetic mutations that cause FV-short overexpression, high TFPI levels and bleeding tendency have been reported. In an attempt to develop a treatment for these disorders, we tried to modulate the expression of FV-short using molecules that target pre-mRNA splicing (morpholino antisense oligonucleotides) or mRNA stability and translation (siRNA). We observed a dose-dependent decrease of FV-short upon treatment with antisense oligonucleotides. Since our findings were observed *in vitro*, more studies are needed to assess safety and efficacy for a possible clinical use. In addition, this molecular approach could be extended to other bleeding disorders where TFPI is the determinant of bleeding severity.

Although our results are still far from a clinical use, these findings contribute to understanding the potential of a molecular therapy and may stimulate further studies investigating this approach in more complex systems in view of a possible future application in patients.

The findings of this thesis have been presented several times at national and international conferences, where they raised interest and stimulated the scientific discussion with other researchers and exchange of ideas and perspectives.

Part of my studies has been carried out using blood of real patients. One of these patients was very enthusiastic about and interested in the research we were conducting on his rare disease. His appreciation and expectations made me realise the societal impact of our work.

CHAPTER 9

Curriculum vitae
List of publications
Acknowledgements

CURRICULUM VITAE

Alice Todaro was born on 5th September 1992 in Monselice (Italy). After completing the secondary education in Classical studies at the "Liceo G.B. Ferrari" High School in Este, she started to study at Ferrara University where she obtained the Bachelor's degree in Biological Sciences and the Master's degree *cum laude* in Biomolecular and Evolutionary Sciences. During the Master internship she worked on coagulation factor IX (Haemophilia B) and focused on rescuing secretion and function of coagulation factor IX impaired by missense mutations through chaperone-like compounds.

Afterwards, she started PhD research at Biochemistry Department of CARIM (Cardiovascular Research Institute Maastricht) at Maastricht University under the supervision of Prof. Tilman M. Hackeng and Dr. Elisabetta Castoldi. The PhD project focused on genetic mechanisms of inherited bleeding disorders as a basis for personalised medicine approaches. In the course of her PhD trajectory she visited the lab of Prof. Paolo Simioni in Padua (Italy) for a research project involving *ex vivo* experiments with megakaryocytes differentiation from haematopoietic stem cells.

During her PhD training she presented her research at national and international congresses and received the "Young Investigator Award" at European Congress on Thrombosis and Haemostasis (ECTH) in 2019; Best poster pitch prize at the Dutch Society of Thrombosis and Haemostasis (NVTH) in 2022; Jeanne Stibbe trophy prize for best oral presentation at the NVTH in 2023.

LIST OF PUBLICATIONS

Papers

- **A.M. Todaro**, C.M. Radu, M. Ciccone, S. Toffanin, M.L. Serino, E. Campello, C. Bulato, B. Lunghi, D. Gemmati, A. Cuneo, T.M. Hackeng, P. Simioni, F. Bernardi, E. Castoldi. *In vitro* and *ex vivo* rescue of a nonsense mutation responsible for severe coagulation factor V deficiency. *J Thromb Haemost* 2023.
- **A.M. Todaro**, T.M. Hackeng, E. Castoldi. Antisense-mediated down-regulation of factor V-short splicing in a liver cell line model. *Appl Sci* 2021.
- A.K. Mohapatra, **A.M. Todaro**, E. Castoldi. Factor V variants in bleeding and thrombosis. *Res Pract Thromb Haemost* 2024.
- S. Pignani, **A. Todaro**, M. Ferrarese, S. Marchi, S. Lombardi, D. Balestra, P. Pinton, F. Bernardi, M. Pinotti, A. Branchini. The chaperone-like sodium phenylbutyrate improves factor IX intracellular trafficking and activity impaired by the frequent p.R294Q mutation. *J Thromb Haemost* 2018.
- **A.M. Todaro**, T.W. van de Berg, J.J.B.C. van Beers, K. Wichapong, T.M. Hackeng, E.A.M. Beckers, Y.M.C. Henskens, F.C.J.I. Heubel-Moenen*, E. Castoldi*. Novel von Willebrand factor propeptide variant (R760S) in a patient with combined type 1/type 2N von Willebrand disease. *equal contribution. In preparation.

Oral and poster presentations

- **A.M. Todaro**, C.M. Radu, M. Ciccone, S. Toffanin, M.L. Serino, E. Campello, C. Bulato, B. Lunghi, D. Gemmati, A. Cuneo, T.M. Hackeng, P. Simioni, F. Bernardi, E. Castoldi. *In vitro* and *ex vivo* rescue of a nonsense mutation (*F5* p.Arg1161Ter) responsible for severe coagulation factor V deficiency. XXXI Congress of the International Society on Thrombosis and Haemostasis (ISTH), Montréal, Canada, June 2023 (oral presentation).
- **A.M. Todaro**, C.M. Radu, M. Ciccone, S. Toffanin, M.L. Serino, E. Campello, C. Bulato, B. Lunghi, D. Gemmati, A. Cuneo, T.M. Hackeng, P. Simioni, F. Bernardi, E. Castoldi. *In vitro* and *ex vivo* rescue of a nonsense mutation (*F5* p.Arg1161Ter) responsible for severe coagulation factor V deficiency. Dutch Society of Thrombosis and Haemostasis (NVTH), Koudekerke, The Netherlands, March 2023 (oral presentation).
- **A.M. Todaro**, M. Ciccone, M.L. Serino, D. Gemmati, A. Cuneo, T.M. Hackeng, F. Bernardi, E. Castoldi. *In vitro* rescue of a nonsense mutation responsible for severe coagulation factor V deficiency using ribosome readthrough agents. XXX Congress International Society on Thrombosis and Haemostasis (ISTH), London, United Kingdom, July 2022 (poster).

- **A.M. Todaro**, M. Ciccone, M.L. Serino, D. Gemmati, A. Cuneo, T.M. Hackeng, F. Bernardi, E. Castoldi. *In vitro* rescue of a nonsense mutation responsible for severe coagulation factor V deficiency using ribosome readthrough agents. Dutch Society of Thrombosis and Haemostasis (NVTH), Koudekerke, The Netherlands, April 2022 (pitch and poster).
- **A. Todaro**, T.M. Hackeng, E. Castoldi. Modulation of alternative splicing of the *F5* gene using morpholino antisense oligonucleotides. CARIM symposium, Maastricht, The Netherlands, November 2019 (poster).
- **A. Todaro**, T.M. Hackeng, E. Castoldi. Modulation of alternative splicing of the *F5* gene using morpholino antisense oligonucleotides. European Congress on Thrombosis and Haemostasis (ECTH), Glasgow, Scotland, October 2019 (oral presentation).

Awards

- Prix d'honneur de la Jeunesse (Young Investigation Award), ECTH Glasgow (UK) 2019
- Best poster pitch prize, NVTH 2022
- Jeanne Stibbe trophy prize for best oral presentation, NVTH 2023
- SimonsFonds grant for conference visit in Montréal (Canada) 2023

Abstracts

- T.W. van de Berg, **A.M. Todaro**, J. van Beers, F. Heubel-Moenen, E. Castoldi, Y.M.C. Henskens, E.A.M. Beckers. A novel quali-quantitative defect of VWF. Dutch Society of Thrombosis and Haemostasis (NVTH), Koudekerke, The Netherlands, April 2022.
- T.W. van de Berg, **A.M. Todaro**, J. van Beers, F. Heubel-Moenen, E. Castoldi, Y.M.C. Henskens, E.A.M. Beckers. Atypical presentation of VWD leading to discovery of novel VWF mutation. International Society on Thrombosis and Haemostasis (ISTH), Philadelphia, United States, July 2021.
- T.W. van de Berg, **A.M. Todaro**, J. van Beers, F. Heubel-Moenen, E. Castoldi, Y.M.C. Henskens, E.A.M. Beckers. A novel quali-quantitative defect of VWF. European Congress on Thrombosis and Haemostasis (ECTH), Gent, Belgium, October 2021.
- T.W. van de Berg, **A.M. Todaro**, J. van Beers, F. Heubel-Moenen, E. Castoldi, Y.M.C. Henskens, E.A.M. Beckers. Atypical presentation of VWD leading to discovery of novel VWF mutation. Dutch Hematology Congress (virtual), January 2021.

- S. Pignani, **A. Todaro**, M. Ferrarese, S. Marchi, S. Lombardi, D. Balestra, P. Pinton, F. Bernardi, M. Pinotti, A. Branchini. The chaperone-like compound sodium phenylbutyrate improves intracellular trafficking, secretion and coagulant activity of factor IX impaired by the frequent p.R294Q mutation. XXV Congresso Nazionale della Società Italiana per lo Studio dell'Emostasi e della Trombosi (SISET), Florence, Italy, November 2018.
- S. Pignani, **A. Todaro**, M. Ferrarese, S. Marchi, S. Lombardi, D. Balestra, P. Pinton, F. Bernardi, M. Pinotti, A. Branchini. A strategy with chaperone-like compounds to restore expression of factor IX variants affected by frequent missense mutations causing Hemophilia B. 23rd Congress of the European Hematology Association (EHA), Stockholm, Sweden, June 2018.
- S. Pignani, **A. Todaro**, M. Ferrarese, S. Marchi, S Lombardi, D. Balestra, P. Pinton, F. Bernardi, M. Pinotti, A. Branchini. Exploring chaperone-like compounds as innovative therapeutic correction approach for factor IX missense mutations causing type I Haemophilia B. XVI Convegno Triennale sui problemi clinici e sociali dell'emofilia - Associazione Italiana Centri Emofilia (AICE), Naples, Italy, November 2017.
- S. Pignani, **A. Todaro**, M. Ferrarese, S. Marchi, S. Lombardi, D. Balestra, P. Pinton, F. Bernardi, M. Pinotti, A. Branchini. The chaperone-like sodium phenylbutyrate improves factor IX intracellular trafficking and activity impaired by the frequent p.R294Q mutation. XXVI Congress of the International Society on Thrombosis and Haemostasis (ISTH), Berlin, Germany, July 2017.

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